Development and characterisation of equine *in vitro* models of respiratory inflammation and resolution

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ABSTRACT

BACKGROUND

Chronic respiratory inflammation is a major cause of recurrent airway obstruction (RAO) in mature horses. RAO has aetiological and clinical similarities to human asthma. Remodelling of airway tissue after bronchial inflammation is evident in RAO and human asthma. Severe asthma in humans is associated with defective lipoxin A4 (LXA4) synthesis and abnormal expression of specific lipoxin receptor (ALX).

Arachidonic acid metabolite LXA4 modulates acute inflammation in a number of species and models of acute inflammation. Dysfunctional LXA4 synthesis and/or expression of the ALX receptor may contribute to the chronic inflammatory response seen in asthma. Abnormal LXA4 and/or ALX expression may also be present in horses with RAO thereby promoting airway remodelling.

HYPOTHESIS

Equine inflammation and its resolution can be modelled *in vitro* using respiratory epithelial and smooth muscle models.

AIMS

To develop an *in vitro* equine respiratory model of respiratory epithelium and airway smooth muscle.

Characterise the response of this model to exogenous lipolysaccharide (LPS) and lipoxin A4 on selected molecules associated with inflammation and inflammatory resolution.

METHODS

Primary equine tracheal epithelial (ETE) cells were obtained from trypsin-dissociation of tracheal epithelial tissue derived from healthy horses with no sign of inflammation at post-mortem. Primary airway smooth muscle (ASM) cells were cultured from explants of equine trachealis muscle.

Near confluent (70-75%) ETE and ASM cells were stimulated with 0.1, 10 and 100μg/ml LPS at 4 and 24 hrs (ETE cells) or 12, 24 and 72hrs (ASM cells). Expression of COX-2 mRNA in these cells was used to determine a suitable time point and LPS concentration to induce inflammation. Inflammatory resolution was then examined by comparing the selected time points and LPS concentrations with the response of ETE and ASM cells to 15 minutes incubation with 100μM LXA4 and LXA4/LPS treatment. Finally, the inflammatory relationship between the epithelium and smooth muscle layer was examined by a co-culture model of ETE and ASM cells. Conditioned media from ETE cells treated with 0.1μg/ml LPS, 100μM LXA4 and LXA4/LPS treatment for 24hrs was used to culture ASM cells for a further 24hrs.

To examine inflammation and its resolution, selected genes, namely ALX, toll-like receptor 4 (TLR-4), tumour-necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were amplified and quantified by real time PCR. ALX and COX-2 proteins were monitored by Western blot and nitric oxide (NO) levels measured by fluorometric analysis. Values for statistical analysis of ETE and ASM cells were obtained with a two and one-way ANOVA and Tukey's multiple comparisons test.

RESULTS

Primary ETE and ASM cells were identified by positive immunocytochemical staining with pan cytokeratin-26 and alpha-smooth muscle actin respectively.

Treatment of ETE cells with 0.1μg/ml LPS for 24hrs increased iNOS and COX-2 mRNA levels, and significantly increased mRNA expression for ALX, TLR-4 TNF-α and IL-1β mRNA (p values <0.05). ASM cells incubated over 72hrs incubation with 0.1μg/ml LPS showed increased expression of selected genes but only significant increases in COX-2 mRNA were observed (p values <0.05).

Incubation of ETE and ASM cells with LXA4 did not significantly increase or inhibit inflammation as measured by real time PCR, Western blot and fluorometric anlaysis. Western blotting showed some inhibition of COX-2 protein in ASM cells but not ETE cells at 72hrs after LXA4 treatment. Fluorometric analysis of NO levels in ETE and ASM cells showed no significant difference after treatment in either cell type. No noticeable evidence of inflammation or inhibition of inflammation was observed in the co-culture model of ETE and ASM cells.

CONCLUSION

It was concluded that an *in vitro* equine model of respiratory epithelium and smooth muscle was successfully established. It was possible to induce partial inflammation in ETE and ASM cells but inflammatory resolution could not be definitively shown in either cell type.

Publications related to thesis

Proceedings of The Physiological Society

University of Manchester (2010) Proc Physiol Soc 19, PC24

"Lipoxin expression in horses with recurrent airway obstruction"

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University of Edinburgh (2011) Proc Physiol Soc 25, C10 and PC10

"Effect of lipoxin A4 on acute inflammation in primary equine airway smooth muscle cells"

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Abbreviations used in thesis

Ahr- Aryl hydrocarbon receptor

AHR-airway hyper-responsiveness

ALI air-liquid interface

ALX/FPRL-lipoxin/formyl peptide receptor-like

AM- alveolar macrophages

ANOVA-analysis of variance

AP-1: activator protein 1

ASM- airway smooth muscle

BAL-Bronchoalveolar lavage

BEAS-2B-human bronchial epithelial cells

BEC- bronchial epithelial cells

BEGM-bronchial epithelial growth medium

bFGF- basic fibroblast growth factor

BLAST- Basic Local Alignment Search Tool

BSA-bovine serum albumin

CD-cluster of differentiation

cDNA- complementary deoxyribonucleic acid

COPD-human chronic obstructive pulmonary disease

COX-2- cyclooxygenase-2

DAPI-4', 6-diamidino-2-phenylindole

DCs-Dendritic cells

DMEM- Dulbecco's Modified Eagle Medium

DNA- Deoxyribonucleic acid

ECL-enhanced chemiluminescence

ECM-extra cellular matrix

EDTA- Ethylenediaminetetraacetic acid

EMBL-EBI-European Molecular Biology Laboratory- European Bioinformatics Institute

eNos- endothelial NOS

ETE-equine tracheal epithelial

FBS-foetal bovine serum

FcεRI- Fc epsilon RI

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF- Granulocyte-macrophage colony-stimulating factor

HRP-horse radish peroxidase

IAD-inflammatory airway disease

ICC-immunocytochemical

IF-immunofluorescent

IFN-γ- Interferon-gamma

IgE-Immunoglobulin

IHC-immunhistochemical

IL-interleukin

IL-1β- interleukin-1 beta

iNOS-inducible nitric oxide synthase

Kb-kilo base pairs

L-15: Leibovitz's 15

LBP-LPS binding protein

LO-lipoxygenase

LPS-lipolysaccharide

LRT-lower respiratory tract

LT-leukotriene

LXA4-lipoxin

MHC-major histocompatibility complex

MIP-2 macrophage inflammatory protein

mRNA- messenger RNA

NCBI- National Centre for Biotechnology Information

nNOS- neuronal nitric oxide synthase

NO-nitric oxide

NOS-nitric oxide synthases

NTC- no template control

OA-osteoarthritis

ONOO-peroxynitrite

p38 MAPK-mitogen-activated protein kinases

PAMP-pattern associated molecular pattern

PBMC-Peripheral blood mononuclear cells

PBS-phosphate buffered saline

PCK-26- Pan-cytokeratin 26

PCR-polymerase chain reactions

Pen/Strep- penicillin/streptomycin

PGE2- prostaglandin E2

Poly IC – Polyinosinic: polycytidylic acid

PRR-pattern recognition receptor

PVDF – polyvinylidene difluoride

q-PCR-real time polymerase reaction

RAO-Recurrent airway obstruction

RIPA- Radio-Immunoprecipitation Assay

RNA-ribonucleic acid

ROS-reactive oxygen species

SAA-serum amyloid A

SDS- sodium dodecyl 19sulphate

SEM-standard error of the mean

SOCS-suppressor of cytokine signalling

SPAOPD-summer pasture-associated airway disease

STDEV-standard deviation

TAE- Tris-acetate-ethylenediaminetetraacetic acid buffer

TA-tracheal aspirate

TBS-Tween-Tris Buffered Saline-Tween

TGF-β transforming growth factor beta

Th-T helper

TLR-Toll-like receptor

Tm-annealing temperature

TNF-α- tumour-necrosis factor alpha

TRAF-TNF Receptor Associated Factor

TUNEL-TdT-mediated dUTP-X nick end labelling

Chapter 1: Immunity and the airways

1.1 Initiation of inflammation

Acute inflammation is an essential part of the immune defence in both the innate and adaptive systems. It is a necessary response to trauma, tissue injury and pathogen infection (Lawrence *et al* 2002). A prompt return to homeostatic equilibrium following acute inflammation ensures minimal damage to surrounding tissue and cells (Han and Ulevitch 2005).

Acute inflammation comprises three key phases:

i) Pathogen recognition and the initiation of inflammation.

ii) Cellular recruitment with synthesis of pro-inflammatory mediators.

iii) Resolution of inflammation.

Disruption of these principal phases can result in progression into chronic inflammation with the development of disease (Henson 2005). During chronic inflammation, both tissue destruction and repair run in parallel in a vicious cycle of host injury.

Chronic inflammation is fundamental to the development of multiple diseases and harmful conditions in humans and in horses. Persistent allergy-induced respiratory inflammation is a feature of both recurrent airway obstruction (RAO) in horses and human asthma.

1.2 Toll-like receptors and the initiation of acute inflammation

Inflammation is triggered after recognition of a wide range of pattern associated molecular pattern (PAMPs), by a group of receptors called

pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are the most influential group of PRRs.

TLRs are transmembrane receptors expressed during innate immune responses and subsequently activate T-lymphocytes in adaptive immunity (Akira and Hemmi 2003). Recognition of invading pathogens by TLRs activates signalling pathways that enable the expression of inflammatory cytokines (Kawai & Akiro 2007). TLRs (**Fig.1**) are stimulated by viruses, bacteria, fungi and helminths, reactive oxygen species (ROS) and by-products from damaged cells (Chaudhuri *et al* 2005).

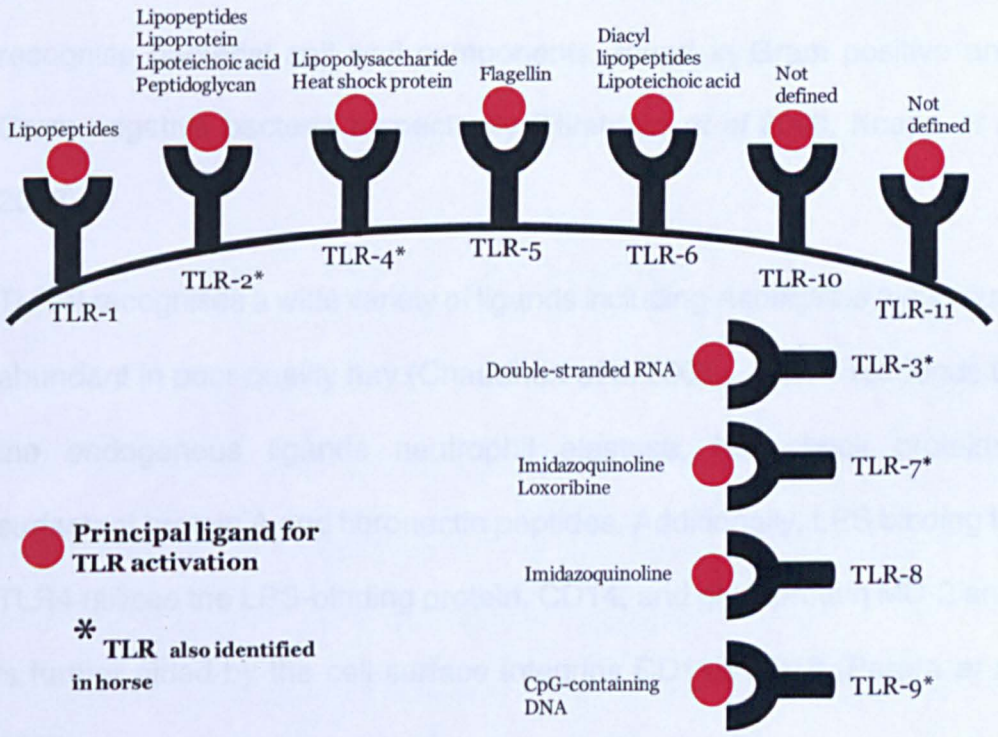

Figure 1: TLRs expressed in human and horse with their principal ligands

(Figure adapted from Akira and Hemmi 2000, and Werners *et al* 2006).

Cell types closely associated with innate defence such as dendritic cells, macrophages, mast cells and respiratory epithelial cells predominantly

express TLRs. Human airway smooth muscle is also known to express functional TLRs (Sukkar *et al* 2006).

The expression of TLR-2, 3, 4, 6, and 9 have been identified in the horse (Gornik *et al* 2011, Quintana *et al* 2011). Evidence of TLR-2 and TLR-4 activity has been found in the lungs of healthy horses (Singh Suri *et al* 2006).

1.3 TLR expression in asthma and pathogen recognition

Excessive binding of TLR-2 and 4 to ligands such as LPS are implicated in the aetiology of asthma (Chaudhuri *et al* 2005). TLR-2 and TLR-4 recognise bacterial cell wall components, found in Gram positive and Gram negative bacteria respectively (Hirshfeld *et al* 2000, Knapp *et al* 2008).

TLR-2 recognises a wide variety of ligands including *Aspergillus fumigatus*, abundant in poor quality hay (Chaudhuri *et al* 2005). TLR-4 responds to the endogenous ligands neutrophil elastase, heat-shock proteins, surfactant protein A and fibronectin peptides. Additionally, LPS binding to TLR4 utilises the LPS-binding protein, CD14, and glycoprotein MD-2 and is further aided by the cell-surface integrins CD11b/CD18 (Perera *et al* 2001).

CD14 is found on cells associated with early inflammatory responses like monocytes, macrophages and neutrophils. CD14 initiates TLR-4 pathogen recognition and triggers TLR-4 to form a homodimer complex with MD-2 (Chow *et al* 1999). Formation of such a complex triggers signalling

pathways (**Fig.2**) leading to the formation of inflammatory products (Barata *et al* 2011)

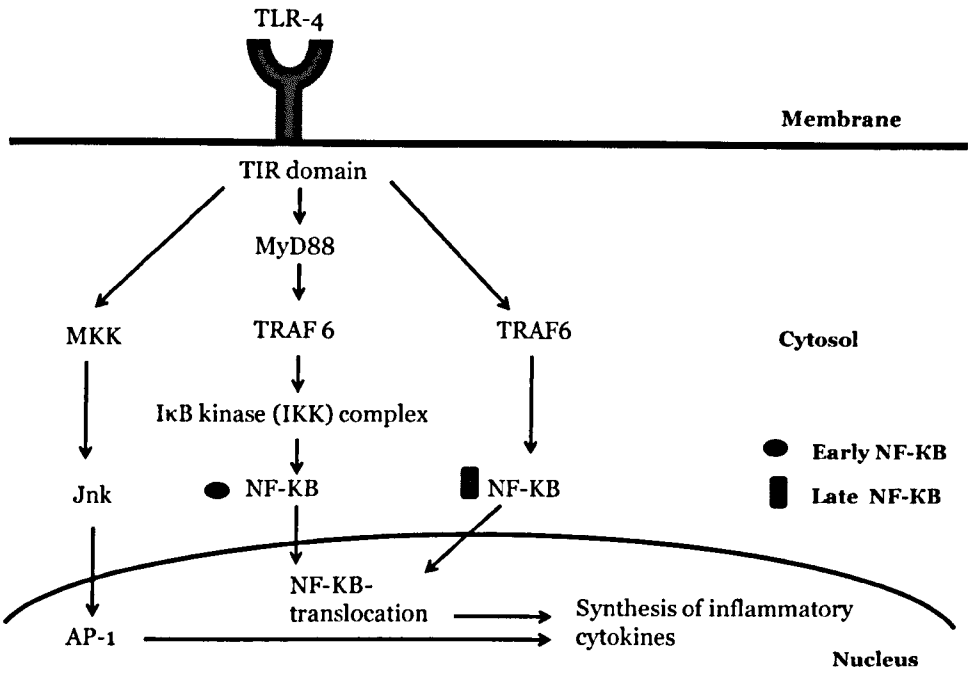

Figure 2: Principal components of TLR-4 signalling pathways

(Figure adapted from Chadhuri *et al* 2005).

1.4 TLR expression in RAO

Exposure to stable dust results in increased TLR-4 mRNA expression in bronchial epithelium with a concomitant increase in IL-8 in horses with RAO (Berndt *et al* 2007). Unusually, TLR-2 mRNA was not increased in bronchial epithelial cells collected from RAO-affected horses after stabling (Berndt *et al* 2009). An 'altered-self' response by TLR-4 may promote inflammation by increased recognition of inflammatory mediators (Greene & McElvaney 2005).

1.5 Defective TLR expression and the onset of allergies

TLR dysfunction can initiate an extreme immune response and cause disease (Atkinson 2008). TLRs actively regulate inflammatory mediator formation and cytokine signalling in airway epithelial cells (Kato and Schleimer 2007, Laberge and Bassam 2004). A point mutation in the human TLR-4 gene (Asp299Gly) may occur in affected individuals (Child *et al* 2003). The Asp299Gly mutation is thought to cause changes to the extra-cellular domain structure (Arbour *et al* 2000). The potential for TLR mutations to cause disease in horses has been investigated. A whole blood assay using 10 healthy horses was used to determine TNF-α expression induced by LPS. A significant alteration of TNF-α production was found in 3 horses with a mutation in their TLR4 cDNA sequence. Despite this, it was not possible to relate this TLR-4 mutation to the altered TNF-α response. The lack of correlation was attributed to differences in TLR-4 expression and the effect of circulating LPS binding protein (LBP) (Werners *et al* 2006).

1.6 TLR activity in early life and allergic disease

The onset of asthma or allergen-related signs of asthma depends on the timing and specific type of allergen exposure. School-aged children who have TLR-4 (Asp299Gly) polymorphisms show an increased prevalence of asthma. These TLR-4 polymorphisms potentially affect the normal early life immune response to environmental pathogens (Fageras-Bottcher *et al* 2003). Dust mites in domestic environments increase the prevalence of childhood asthma (Liu 2002). In contrast, agricultural environments are associated with a decrease in childhood asthma or airway hyper-

responsiveness (Liu 2002). This decrease is attributed to frequent animal exposure and early life exposure to stables or barns (von Ehrenstein 2000, Riedler *et al* 2001).

The effect of exposure in early life to environmental dust and allergens in horses is unknown. Hotchkiss *et al* (2007) performed a questionnaire using a sample of horse owners in Great Britain. The questions related to management and exposure to known RAO risk factors. The study showed that before five years of age, 67.5% of 380 horses had been exposed to straw, and 14.5% of those had experienced respiratory infection of more than a few days. However, the report relies on largely anecdotal evidence, with no description of length of time for exposure, the degree of stabling experienced by the horses or the proportion that went on to develop RAO in later life.

1.7 Cytology of the airways during acute pulmonary inflammation

Excessive TLR expression is shown to be important in both the development of allergic respiratory disease and the promotion of acute inflammation. Once acute inflammation is initiated, cellular recruitment and the synthesis of pro-inflammatory products occurs.

Airway inflammation in RAO and asthma is a complex interaction between different cell types. In addition to mast cell and T-lymphocyte activity, sentinel cells (dendritic cells, macrophages, and neutrophils) actively promote or initiate inflammation. (Pietra *et al* 2007). Macrophages, resident dendritic cells and epithelial cells are particularly primed to activate inflammation. Increased eosinophils in circulation are found in mild

to moderate asthma, whereas a high neutrophil count (>25%) is more prevalent in RAO. Severe human asthma is now increasingly associated with neutrophilic inflammation (Sur *et al* 1993, Kamath *et al* 2005, Turato *et al* 2007).

In contrast, samples of BAL fluid (200-500ml of saline) from RAO-free animals contain predominantly alveolar macrophages (60%) and lymphocytes (35%), with the remainder a mixture of neutrophils, mast cells and epithelial cells (Lavoie 2007). Respiratory epithelial and airway smooth muscle cells are now recognised as forming part of the inflammatory response and for the role they play in airway remodelling.

Dendritic cells

Dendritic cells (DCs) are key sentinel cells for pathogen recognition and initiating inflammation. Dendritic cells present antigen for processing by T-cells which can trigger Th1 or Th2-type responses (Hammad & Lambrecht 2008). DCs may initiate excessive Th2 immune responses to allergens (Lambrecht & Hammad 2011). Equine dendritic cells have been successfully isolated and identified using morphology and immuno-staining for MHC class II (Siedek *et al* 1997), as activated dendritic cells show MHC II upregulation.

Innate immune responses and DCs' antigen presentation is directly influenced by their activation state (Cavatorta *et al* 2009). DC involvement is seen in both a compromised immune system as well as respiratory and allergic disease. Work investigating equine DCs has predominantly focused on their role in bacterial diseases, particularly *Rhodococcus equi*

infection in foals (Flaminio *et al* 2009). Other investigations (Dietze *et al* 2008) indicate that LPS and IL-4 can affect DCs' maturation in horses.

However, neither LPS nor the synthetic immunostimulant polyinosinic: polycytidylic acid poly (I: C) alone are considered sufficient for full DCs maturation (Mauel *et al* 2006). Additionally, human airway epithelial cells are shown to directly influence DCs' activity (Lambrecht & Hammad 2011). Despite this, no substantial evidence of a role for DCs' in the development of RAO exists.

Macrophages

Macrophages play a prominent role in antigen presentation during immune responses. Alveolar macrophage (AM) activity may contribute to sensitivity to LPS-induced endotoxemia in horses (Parbhakar *et al* 2004, Hammond *et al* 1999).

Alveolar macrophages from RAO-affected horses show increased expression of TNF-α, IL-1β and IL-6 after exposure to an LPS and hay dust suspension (Laan *et al* 2006). Equine alveolar macrophages have been shown to release IL-8 and macrophage inflammatory protein (MIP-2), both potent chemokines for neutrophil activity and recruitment (Laan *et al* 2006).

Evaluation of alveolar macrophages' influence on neutrophil recruitment and influx during respiratory disease was investigated over a period of 2 years (Joubert *et al* 2011). Macrophages were not thought to be the principal cell type causing neutrophil influx despite expression of neutrophil chemoattractants IL-8 and MIP-2.

AM activity is attributed to the airway remodelling observed in human chronic obstructive pulmonary disease (COPD) rather than asthma (Aoshiba & Nagai 2004). Pulmonary responses in horses induced by LPS were compared with and without pulmonary macrophages. Reduction of pulmonary macrophages was achieved by the use of intravenous gadolinium chloride, a macrophage inhibitor. Depletion of pulmonary macrophages reduced but did not eliminate LPS-related inflammation (Parbhakar *et al* 2005). This suggests that their role is contributory, but not a dominating factor in airway inflammation and airway remodelling.

Neutrophils

Early influx of neutrophils and their activation is a distinguishing feature of chronic inflammation in RAO. Airway neutrophil numbers are greater in RAO-affected horses when compared with normal horses (Brazil *et al* 2005, Franchini *et al* 2000). Following an investigation of the potential variation in BAL fluid samples from horses with RAO, it was estimated that the proportion of neutrophils was over 25% in all samples (Jean *et al* 2011).

Neutrophil migration to the airways is recorded in horses with clinical signs of RAO after 5 hours of mouldy hay/straw challenge. Neutrophils persist in the airways if exposure to organic dust and mould continues, with apoptosis delayed by LPS exposure (Brazil *et al* 2005). Continued neutrophil activity, inhibition of apoptosis, or ineffective neutrophil removal perpetuates chronic inflammatory conditions (El Kebir *et al* 2008).

Neutrophils release a combination of damaging substances in RAO; proteases, nucleases, peroxides, nitric oxide and reactive oxygen species

(Franchini *et al* 1998). Reactive oxygen species are linked to airway disorders, including asthma by promoting respiratory inflammation (Henricks & Nijkamp 2001).

The role of neutrophils in allergic airway inflammation is controversial. Morphological changes were induced by IL-4 in neutrophils with Th2 type lung inflammation related to neutrophil activity (Lavoie-Lamoureux *et al* 2010). In contrast, no difference was found in the expression of mRNA for TNF-α and IL-1β in peripheral blood neutrophils from RAO-affected horses and control groups (Joubert *et al* 2008). The authors considered these results indicated that such neutrophils were unlikely to contribute to the development of RAO.

Neutrophils are also recruited by other cell types, typically those prominent in pathogen recognition. TLR-4 mRNA is increased in bronchial epithelial cells from horses with RAO, with the subsequent production of IL-8, a potent chemokine for neutrophils (Berndt *et al* 2007). The European Network for Understanding Mechanisms of Severe Asthma (2003) considers severe asthma to have a phenotype characterised by a neutrophilic infiltrate. Additionally, other work suggests predominance of neutrophil chemokine IL-8 in severe asthma (Planaguma *et al* 2008, Wenzel *et al* 2006, and Holgate *et al* 2006).

Neutrophils are undoubtedly a significant contributory element during acute airway inflammation. A continued neutrophil presence, either through prolonged antigen exposure or a failure of neutrophil apoptosis and removal is noted as part of chronic inflammation (Hughes *et al* 2011).

However, neutrophil recruitment is secondary to inflammation rather than acting as the prime mediator of it.

Therefore, other cell types in the airways must be capable of initial pathogen detection, initiation of acute inflammation, and promotion of chronic inflammation should dysfunction or irregularities occur. Human bronchial epithelial cells are known to be active in all these respects. Such cells are also able to direct the migration and actions of monocytes, and T-lymphocytes (Mayer *et al* 2007). Human bronchial cells are described as having the capacity to create a "microenvironment" and actively direct immunity (Altraja *et al* 2009).

Airway epithelial cells

Airway epithelial cells directly interact and influence other immune cells and so orchestrate both the innate and acquired immune response (Hamilton *et al* 2001, Holgate *et al* 2009). Stimulated human airway epithelial cells can release high concentrations of biological mediators that perpetuate inflammation and bronchial hyperesponsiveness (Takizawa 1998, Schleimer 2004).

The equine respiratory epithelium is also suggested to be a source of such mediators during early inflammatory reactions (Ainsworth *et al* 2003). The biological mediators generated by the epithelium are broadly divided into cytokines, chemokines, enzymes and eicosanoids and include: TNF-α, IL-1β, IL-8, IL-6, COX-2, lipid mediators and nitric oxide (Knight & Holgate 2003, Polito & Proud 1998).

Airway smooth muscle cells

Airway smooth muscle (ASM) is an additional cell type in the airways that plays an active part in immune and inflammatory regulation (Ozier *et al* 2011). ASM is the primary determinant of airway resistance (Robinson 2007). Bronchospasm of ASM is exacerbated by elevated nitric oxide (NO) concentrations (Matera *et al* 2002).

The role of airway smooth muscle in the pathophysiology of RAO and asthma is associated with increasing airway hyperesponsiveness and narrowing of the airways. ASM is also a source of inflammatory mediators, particularly once the disease has entered a chronic phase (Hirst 1992). This is typically once remodelling of the airways has been instigated.

1.8 Pro-inflammatory mediators generated during acute airway inflammation

Cytokines

Tumour necrosis factor alpha (TNF-α)

TNF-α has roles in the instigation of acute and chronic inflammation and acts as a mediator responsible for airway remodelling (Cho 2011). As such, the expression of TNF-α is frequently used as marker of inflammation. Suppression of TNF-α in people is considered to be a target for disease treatment (Tracey & Cerami 1994). The influence of TNF-α is widely studied in equine conditions such as osteoarthritis, gastro-intestinal (GI) disturbances, and insulin resistance.

In contrast, the role of TNF-α in equine airway inflammation or RAO in horses has received relatively little consideration in comparison to human asthma. TNF-α expression in circulating blood is readily induced in horses

performing severe or high intensity exercise (Liburt *et al* 2010). Equine peripheral leucocytes show increased expression of mRNA for TNF-α in response to inhaled LPS (Sykes *et al* 2005). An increase in TNF-α mRNA expression has been demonstrated in equine vascular smooth muscle, and considered to be an indicator of early endotoxemia (Rodgerson *et al* 2001). Unusually, TNF-α incubation of equine monocytes did not induce TLR-2 or TLR-4 activation suggesting that although TNF-α is vital in the initiation of signalling pathways, it does not influence TLR activation (Kwon *et al* 2010).

RAO-affected horses during exacerbation show increased TNF-α levels despite clinical resolution following treatment with anti-inflammatory glucocorticoid fluticasone (Giguere *et al* 2002). Similarly, RAO-affected horses still have detectable levels of TNF-α in serum despite the absence of clinical signs. This suggests that current anti-inflammatory treatment fails to fully resolve TNF-α induced inflammation (Lavoie-Lamouroux *et al* 2012).

Interleukin-1 beta (IL-1β)

In a similar manner to TNF-α, the expression of IL-1β is frequently used as a biomarker of early or acute inflammation. Expression of IL-1β mRNA in horses is rapid with a considerable fold concentration change following LPS exposure or incubation (Ainsworth & Ryner 2012). Horses treated with intravenous doses of LPS (total dose, 80 μg), developed systemic inflammation, with IL-1β expression strongly associated with expression of cytokines IL-6 and IL-8.

IL-1β in people has been correlated with inducing COX-2 and iNOS expression, both strongly associated with human airway disease (Redinton *et al* 2001). COX-2 mRNA expression was correlated to IL-1β levels in equine smooth muscle cells after LPS incubation (Rodgerson *et al* 2001). Horses with inflammatory airway disease (IAD) also show significantly increased levels of IL-1β mRNA in cell pellets obtained from BAL samples (Hughes *et al* 2011).

In contrast, bronchial alveolar lavage (BAL) samples from RAO-affected horses after 24 hours of dusty hay had neutrophils removed. Cell samples were treated with LPS solutions, but this treatment did not significantly increase IL-1β levels (Reyner *et al* 2009). The complexities of IL-1β expression, and its interaction with TNF-α, during respiratory inflammation are not fully understood in the horse.

Chemokines

Interleukin 8 (IL-8)

IL-8 has been subject to more recent investigation as it is produced by several cell types including neutrophils, endothelial cells, epithelial cells and smooth muscle cells. Neutrophil number and lung dysfunction in asthma has been associated with a corresponding increase in IL-8 levels (Pease and Sabroe 2002). Previous studies have demonstrated evidence of increased expression of IL-8 mRNA from BAL fluid and epithelium of RAO-affected horses (Franchini *et al* 2000, Ainsworth *et al* 2003, 2006, Laan *et al* 2006 Berndt *et al* 2007, and Riihimaki *et al* 2008b).

Interleukin 6 (IL-6)

During acute inflammation, epithelial cells synthesise IL-6, a cytokine with pleiotropic qualities. IL-6 can increase neutrophil activation, recruitment and adhesion during migration, as well as stimulating Th2 cells (Horohov *et al* 2005). BAL fluid from RAO-affected horses has elevated IL-6 mRNA concentrations (Pietra *et al* 2007, Riihimaki *et al* 2008).

Enzymes

Cyclooxygenase (COX)-2

The enzyme cyclooxygenase has two distinct isoforms. COX-1 is expressed constitutively and COX-2 is synthesized during inflammation (Blikslager 2006). COX-2 is an important mediator of prostaglandins, noted for their role in asthma pathogenesis.

Prostaglandins, thromboxanes and leukotrienes are pro-inflammatory eicosanoids generated from arachidonic acid metabolism (Serhan *et al* 2008). COX-2 expression enables production of prostaglandin E_2 (PGE_2). Elevated PGE_2 concentrations induce bronchoconstriction and Th2 subset differentiation (Kalinski *et al* 1997). Epithelial expression of COX-2 is significantly increased in human patients with asthma (Sousa *et al* 1997). Acute inflammatory models using equine leukocytes show LPS-induced expression of COX-2 mRNA and COX-2 proteins (Stewart *et al* 2009, Eckert *et al* 2007). *In vitro*, equine vascular smooth muscle cells also synthesise COX-2, in response to endotoxin exposure (Janicke *et al* 2003, Rodgerson *et al* 2001).

The importance of COX-2 expression in the pathogenesis of RAO is largely unresolved; however, COX-2 may have a beneficial role in the subsequent generation of anti-inflammatory mediators. COX-2 is taken up

by monocytes, endothelial cells or airway epithelial cells for the synthesis of lipoxins (Brezinski & Serhan 1990). COX-2 uptake by these cells may be significant in preventing the development of chronic inflammation.

Nitric oxide (NO)

NO is a complex and diverse molecule with both beneficial and harmful actions (Bogdan 2001). NO regulates vascular tone and aspects of immunity and inflammation (Hayward *et al* 1999).

NO production is reported to vary within species, and according to the inciting stimulus (Aktan 2004). NO production relies on the oxidation of L-arginine, mediated by a group of enzymes, the nitric oxide synthases (NOS), either membrane-bound (eNOS), or soluble (nNOS, iNOS) (Aktan 2004).

Increased synthesis of NO and its metabolites is considered to promote airflow limitation and the pathogenesis of respiratory disease. This includes asthma, COPD, and cystic fibrosis (Hayward *et al* 1999, Knight & Holgate 2003, Ricciardolo *et al* 2006). Numerous cell types synthesize NO but increased iNOS production is prevalent in airway epithelial and airway smooth muscle cells (Karpuzoglu & Ahmed 2006). The synthesis of NO by human airway epithelial cells is considered to suppress Th1 cytokine production. This enhances Th2 subset activity and contributes to allergic inflammation (Barnes & Liew 1995). Expression of the NO metabolite peroxynitrite (ONOO-) may promote the continued recruitment of neutrophils (Jozsef *et al* 2002).

1.9 Lipoxins as pro-resolution and anti-inflammatory mediators

Moderation of the inflammatory process

Acute inflammation is a necessarily self-limiting process to prevent excessive tissue damage. Several mechanisms signal the change from pro-inflammation to active resolution of this process (Haworth & Buckley 2007, Serhan 2007). The end of inflammation is indicated by synthesis of pro-inflammatory leukotrienes and prostaglandins switching (**Fig.3**) to the formation of pro-resolution mediators, namely resolvins, protectins and lipoxins. Resolvins and protectins are associated with promoting tissue repair, and lipoxins represent the pivotal point in ending inflammation (Medzhitov 2008).

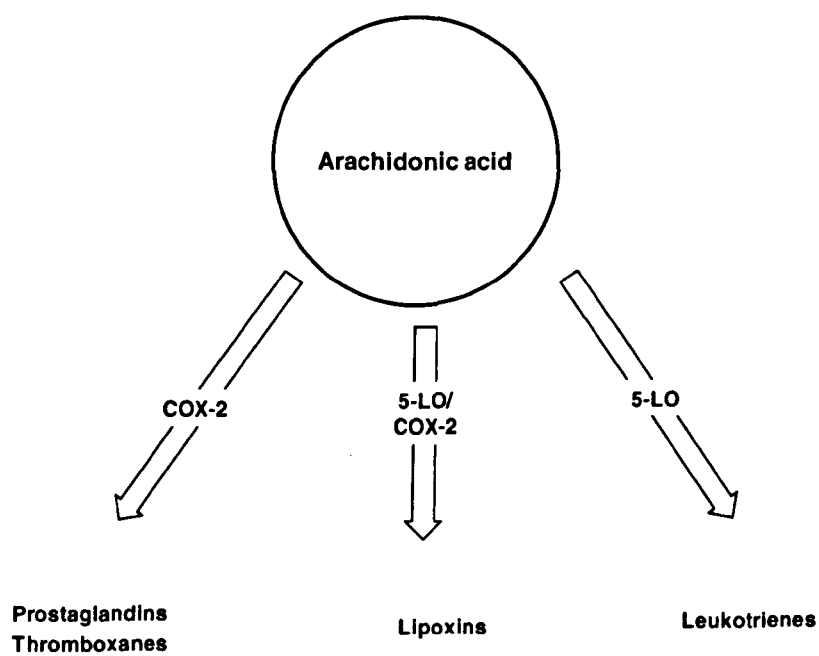

Figure 3: Arachidonic acid metabolites and pathways for synthesis

(Figure adapted from Serhan 2007)

Lipoxins as pro-resolution agents

Lipoxin production is observed in several species, including rat, mouse, fish and humans (Serhan 2005). Synthesis occurs *in vivo*, mainly through cell-cell interaction and predominantly uses three specific pathways acting separately or jointly (Rowley *et al* 1994). Single cell production of lipoxins can occur, as demonstrated in activated leukocytes from asthmatics after exposure to inflammatory cytokines (Chavis *et al* 1995).

The cells involved in lipoxin synthesis include neutrophils, airway epithelial cells, platelets, monocytes, macrophages, eosinophils and vascular endothelial cells. Synthesis occurs when arachidonic acid is taken up by these cells (**Fig.4**) and converted to either LXA4 or LXB4 through the enzymatic action of lipoxygenases (LO) 5-LO, 12-LO, and 15-LO (Brezinski and Serhan 1990).

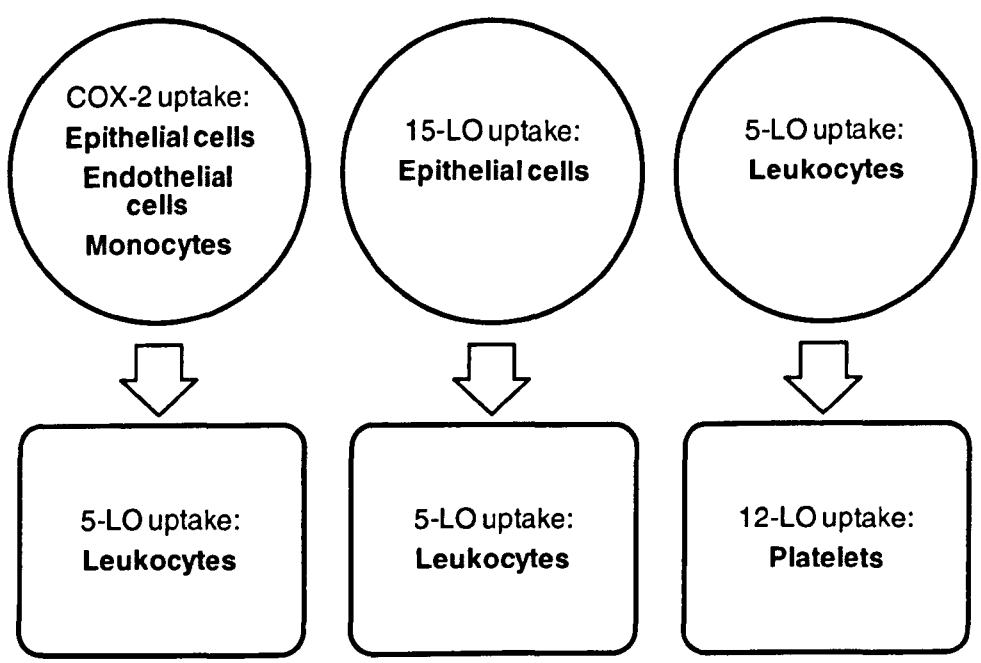

Figure 4: Pathways and cells involved for synthesis of lipoxins

(Figure adapted from Brink *et al* 2003)

Lipoxin receptors

Lipoxins bind with high affinity to the receptors designated ALX and Ahr. ALX is a G-protein-coupled receptor (GPCR) originally designated formyl peptide receptor-like 1 (FPRL-1). Current nomenclature favours the term ALX, or ALX/FPRL-1 based on the high affinity binding and selectivity displayed by this receptor for LXA4 (Brink *et al* 2003). Lipoxins also signal through the ligand-activated aryl hydrocarbon receptor (Ahr), a nuclear receptor from the superfamily of DNA-binding proteins (Schaldach *et al* 1999).

Cell types that exhibit ALX receptors are mainly leukocytes, but lipoxins regulate macrophages, dendritic cells and affect Th1 and Th2 expression (Parkinson 2006). The main organs of expression are the spleen and lung but also include heart, placenta and liver.

LXA4 is the most potent naturally occurring ligand for ALX but other lipid ligands are able to activate this receptor, namely 15-epi-LXA, ATLa1 and ATLa2. There is also partial competition from LTC4, LTD4, fMLF and 15-deoxy-LXA4 indicating that ALX is capable of binding to several ligands (Brink *et al* 2003).

Lipoxin modulation of inflammation

Lipoxins may be capable of anti-inflammatory roles by modulating activity, signalling pathway intermediates and inhibiting inflammatory cytokine production (Machado *et al* 2008). Inhibition of TLR activity occurs when Ahr signalling causes degradation of TLR signalling intermediates, TNF Receptor Associated Factor (TRAF) 2 and 6 (Machado *et al* 2008).

LXA4 analogues also inhibit cellular signalling by preventing nuclear accumulation of AP-1 and NF-κB in polymorphonuclear and mononuclear leukocytes (Jozsef *et al* 2002). Inhibition of NF-κB and AP-1 activity subsequently decreases expression of the neutrophil chemo-attractant ONOO (Filep *et al* 2005). The principal effect of LXA4 binding to ALX in relationship to the pathways generating inflammatory molecules is shown in **Fig 5.**

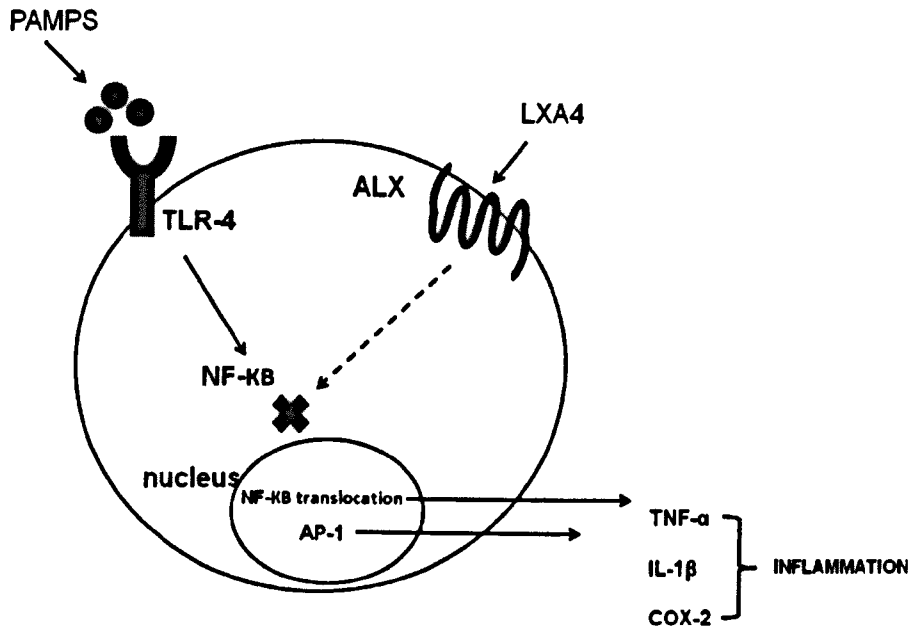

Figure 5: Diagram of principal effects of LXA4 on inflammation

(Figure adapted from Serhan 2008)

Dysfunctional lipoxin synthesis and chronic disease

Inadequate or dysfunctional lipoxin synthesis is implicated in chronic inflammation and disease. Asthma severity is associated with decreased or defective capacity for LXA4 production (Celik *et al* 2007). Whole blood analyzed from severely asthmatic patients revealed low concentrations of LXA4 (Planaguma *et al* 2008). A similar lack of LXA4 in sputum collected

from equally asthmatic subjects was accompanied by increased concentrations of LTB4 and IL-8.

In contrast, patients with mild asthma had increased concentrations of LXA4 in their sputum (Vachier *et al* 2005). Interestingly, dexamethasone treatment inhibited LXA4 expression in severe asthmatics compared with non-severe asthmatics (Bhavsar *et al* 2010). This suggests that the anti-inflammatory effects of dexamethasone may prevent full resolution of acute inflammation and thus promote chronic inflammation. Additionally, LXA4 production appears to be defective in cystic fibrosis (CF), a pulmonary disease characterised by neutrophil-mediated inflammation, persistent infection and tissue damage. (Karp *et al* 2004). Dysfunctional expression of ALX may also increase the effect of acute serum amyloid A (SAA). SAA is a protein that signals through ALX and has the capacity to modulate or promote inflammation (El Kebir *et al* 2008).

Exercise-induced asthma elicits airway hyper-responsiveness and bronchoconstriction. Asthmatic children have decreased levels of plasma LXA4 after exercise with LXA4 levels related to the severity of asthma (Tahan *et al* 2008). Similarly, horses with RAO frequently exhibit such exercise intolerance (Davis *et al* 2005, Lykkesfeldt & Svendsen 2007), which may be related to low LXA4 levels.

The cause of defective synthesis of LXA4 and loss of ALX expression in severe asthma is currently poorly understood (Celik *et al* 2007). However, if lipoxin synthesis is restricted in severe neutrophil-dominated asthma, then lipoxin synthesis in horses with RAO may be similarly diminished.

1.10 Recurrent airway obstruction

Recurrent airway obstruction (RAO) in horses is classified as chronic lower airway inflammation, caused by the environment in which stabled horses are kept (Robinson *et al* 1996). RAO has previously been thought to be similar to human chronic obstructive pulmonary disease (COPD), and was previously described as equine COPD. Human COPD differs from RAO in its aetiology and pathogenesis and so this term is now considered inaccurate.

The phrase 'Recurrent airway obstruction' has been proposed to define mature horses with reversible airway obstruction arising from environmental challenge. RAO is thought to be caused by an allergic reaction to irritants entering the respiratory tract such as allergens, dust or pollen (Robinson 2001).

Clinical signs and epidemiology of RAO

Clinical signs of RAO become apparent in horses frequently described in the literature as 'middle-aged'. The mean age of onset has been calculated as 12.4 years (s.d.± 6.8 years) (Hotchkiss *et al* 2007). Horses with RAO commonly present with airway hyper-responsiveness (AHR), bronchospasm, mucus accumulation, and airway inflammation.

RAO has been estimated to affect half of all horses worldwide (Bowles *et al* 2002). Susceptible individuals may have a genetic predisposition to acquiring the condition (Gerber *et al* 2008, Jost *et al* 2007). The risk of developing the condition is increased in the offspring of RAO-affected parents (Marti *et al* 1991, Ramseyer *et al* 2007). Neither breed nor gender influences the risk of the development of RAO.

Clinical differentiation of RAO from other equine respiratory diseases

Certain respiratory disorders in the horse may initially present with generic signs of airway disease (**Table 1**).

Airway disorders	Reduced Airflow	Increased inflammation
Upper airway disorders	✔	
Dorsal displacement of soft palate	✔	
Laryngeal hemiplegia	✔	
Pharyngeal collapse	✔	
Sinusitis		✔
Epiglotitis		✔
Lower Airway disorders		
Inflammatory airway disease		✔
RAO	✔	✔

Table 1: Common airway disorders in the horse where reduced airflow and increased inflammation are present

(Adapted from Lavoie 2007)

Confirmation of inflammatory airway disease (IAD) in poorly performing sports horses that show increased levels of mucus and inflammatory cells in the LRT (Hughes *et al* 2011) requires careful evaluation to distinguish it from RAO (**Table 2**).

Category	RAO	IAD
Age of onset	12.4 years	Any age
Respiratory changes	Respiratory distress including at rest	Respiration relatively unaffected
Bronchial alveolar lavage cytology	Neutrophil count >25%	Neutrophil count < 20%

Table 2: Clinical differentiation between IAD and RAO in horses

(Adapted from Couetil *et al* 2007 and International Workshop on Equine Chronic Airway Disease 2001)

Differential diagnosis of RAO is based on the clinical history of the affected animal, a clinical examination, and interpretation of diagnostic tests (**Table 3**) (Couetil *et al* 2007).

Diagnostic test	Method of evaluation
Respiratory function	Clinical scoring of cough, nasal discharge and abdominal effort
Degree of airway obstruction	Respiratory mechanics for evaluation of lung function : Measurement of flow rates, forced oscillation techniques, forced expiration respiratory inductance plethysmography
Gas exchange	Arterial blood gas analysis of arterial hypoxemia
Pulmonary inflammation	Exhaled breath condensate, BAL samples, bronchial brushings and lung biopsy
Imaging methods	Endoscopy and radiography for signs of mucopus and lung changes

Table 3: Diagnostic tests for RAO

(Information from Equine Respiratory Medicine and Surgery (Lavoie 2007)

Bronchoalveolar lavage (BAL) cytology is considered representative of lung histopathology (Hoffman 2008) with its results frequently used to confirm a diagnosis of RAO. Total cell counts recovered from BAL fluid containing >25% neutrophils indicate an RAO-associated airway obstruction (Robinson 2001). In contrast, neutrophil numbers in BAL fluid of unaffected horses represent <10% of total cell count (Couetil *et al* 2001).

Welfare implications for horses with RAO

Clinical manifestation of RAO in horses becomes increasingly apparent as the disease progresses. Mild to moderate RAO-affected animals can be free from clinical signs and remain bright with good appetite. Signs of mild RAO may include slight or modest exercise intolerance.

In severe cases, the health and welfare of the animal may be compromised. Such animals display visible respiratory distress, often at rest (Robinson 2001). Horses with RAO can deteriorate rapidly and experience severe inhibition of their athletic capabilities.

The prognosis for a return to normal health in horses with RAO is poor. Episodes of exacerbation in RAO include bouts of severe non-productive coughing and periods of dyspnoea. During such periods a double expiratory effort may be required to maintain adequate ventilation. Advanced cases of RAO develop hypertrophy of the external abdominal oblique muscle from the respiratory exertion. This appears as a visible external line and has led to the colloquial description of RAO as 'heaves' (Robinson 2001).

Available management and treatment of the disease

Current management of RAO requires stringent control of environmental irritants accompanied by anti-inflammatory medication (**Table 4**). Treatment is limited to symptomatic relief (Cunningham & Dunkel 2008).

Medication	Principal effect and drug mechanism
Corticosteroids e.g. Dexamethasone/Beclommethasone/Fluticasone	Anti-inflammatory group of glucocorticoid steroids. The principal action is to bind to the glucocorticoid receptor which prevents translocation of transcription factors into the cytosol to inhibit the production of inflammatory proteins
Bronchodilators e.g. Clenbuterol/Salmeterol/Pirbuterol	Improve breathing by reducing airway obstruction and mucus accumulation. Bronchodilators act on β_2-adrenergic receptors to relax smooth muscle and enable dilation of bronchial airways

Table 4: Principal treatments available for symptomatic control of RAO

(Adapted from Lavoie 2007)

Despite careful management and medication, clinical signs of RAO can take several weeks to subside (Dixon et al 1995). RAO remains a poorly understood disease with high welfare and financial implications. For these

reasons, further investigation of RAO and its underlying mechanisms is warranted.

Similarities between human asthma and RAO

Aspects of the aetiology, pathogenesis and clinical signs of RAO are comparable with human asthma. Asthma is described as a narrowing of the airways following contact with an irritant. Symptoms of asthma include shortness of breath, bouts of coughing, and a tight feeling in the chest (Wenzel 2006).

Clinical similarities of RAO to asthma

Equine RAO and asthma in humans both show varying degrees of pulmonary dysfunction affecting airflow and respiration. Either condition can present with airway hyper-responsiveness (AHR), bronchospasm, mucus accumulation, and airway inflammation (Robinson 2001, Chipps *et al* 2012).

Airway hyperesponsiveness

AHR is triggered by multiple factors and may subsequently provoke bronchospasm. Bronchospasm induces bronchoconstriction, with coughing and dyspnoea (Robinson 2001, Suguikawa *et al* 2009). Variable airflow obstruction in RAO and asthma is reversible either spontaneously or with intervention and altered clinical management. Clinical management frequently requires severely limiting exposure or removal of the principal allergen (Suguikawa *et al* 2009).

Mucus accumulation

Persistent mucus accumulation perpetuates the bronchoconstriction present in asthma and RAO. Excessive mucus production is correlated with lung dysfunction and damage (Murdoch & Lloyd 2010).

The exact cause of asthma is unclear but several factors increase the likelihood of its development. This includes genetic predisposition such as a family history of allergy and environmental influences including smoking and pollution. Asthma may develop at any age with several key triggers **(Fig.6)** capable of initiating an exacerbation (Chipps *et al* 2012).

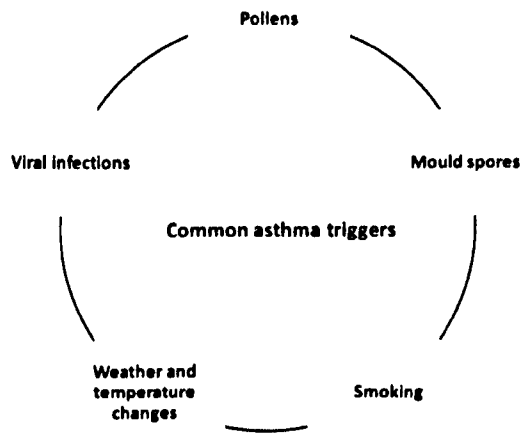

Figure 6: Common asthma triggers

(Adapted from Chipps *et al* 2012)

The pathogenesis of asthma is associated with the multi-cellular action of mast cells, eosinophils, neutrophils and $CD4^+$ T-lymphocytes (Holgate 2008). Severe asthma sufferers are therapy resistant and experience increasingly worsening symptoms (Haselkorn 2009). Human asthma has well documented origins in allergy as a Type 1 hypersensitivity reaction (Holgate 2011). Evidence for the role of type 1 hypersensitivity in the pathogenesis of RAO is equivocal.

1.11 Evidence for RAO as an allergic disease

Type I hypersensitivity

Evidence outlining a Type 1 hypersensitivity reaction as the pathogenic basis for RAO is divisive (Wagner 2009). Type I hypersensitivity immune

reactions occur in people and are frequently initiated by environmental factors such as pathogens or diet. Immunoglobulin E (IgE) concentrations are frequently elevated in individuals with type I hypersensitivity.

Antigen exposure can cause IgE-bound high-affinity FcεRI receptors expressed on mast cells to cross-link. Cross-linking initiates degranulation of mast cells and the release of molecules such as histamine, heparin, and eicosanoids which initiate clinical signs of allergy and inflammation (Tizzard 2004).

Clinical signs of Type 1 hypersensitivity in atopic or sensitized humans present with an early and late phase response. Early phase reactions occur within minutes of allergen exposure with late phase responses peaking at 6-12 hours before subsiding (Tizzard 2004).

Allergen testing in horses

IgE-mediated allergies in horses such as insect bite hypersensitivity are well documented (Wagner 2009). Intra-dermal skin tests to measure immune reactivity to IgE indicative of Type 1 hypersensitivity yield inconclusive results in RAO-affected horses. Results showed considerable variability, with positive results recorded in control groups, and repeatability of tests proving questionable (Lebis *et al* 2002, Tahon *et al* 2009). Data from dermal allergy testing has not indicated significant early phase IgE mediated development of RAO. Evidence for both early and late phase hypersensitivity in horses with RAO is unclear

Early phase hypersensitivity in RAO

Early studies suggested inhalation of allergens *Faenia rectivirgula* sp. and *Aspergillus fumigatus* sp. induced type I hypersensitivity responses

(McGorum *et al* 1993, Robinson 1996). More recent works demonstrate that serum from RAO horses has increased IgE levels, specifically in response to recombinant mould *A. fumigatus* 8 (rAspf8) (Kunzle *et al* 2007).

Data confirming mast cell activity in RAO as an indicator of early stage hypersensitivity is limited. Allergen challenges using suspensions of hay/straw dust increased BAL fluid histamine concentrations in control horses, but not in RAO-susceptible horses. Similar studies found BAL fluid from RAO-affected horses had increased histamine concentrations only after several hours of inhalation challenge (McGorum *et al* 1993, Deaton *et al* 2007,). Despite the development of antibodies to detect IgE and assays to quantify total serum IgE levels, early stage assessment of RAO remains controversial (Wagner 2009).

Late phase hypersensitivity in RAO

Clinical signs of RAO can be induced 6-12 hours after allergen challenges such as dry/mouldy hay or stabling on straw bedding. This is associated with a Th2-type response indicating late phase hypersensitivity (Lavoie *et al* 2001, Cordeau *et al* 2004).

Divergence of undifferentiated CD4+ T helper cells (ThO) into Th1 (cell mediated immunity) or Th2 (humoral immunity) subsets is determined by the cellular environment (Opal and DePalo 2000). Following activation, by antigen recognition and co-stimulation, Th1 or Th2 cells undergo clonal expansion and express cytokines specific to that cell subset (Tizzard 2004). Th1 cells are characterised by production of IFN-γ, while Th2 cells produce IL-4, -5 and -13 (**Fig.7**)

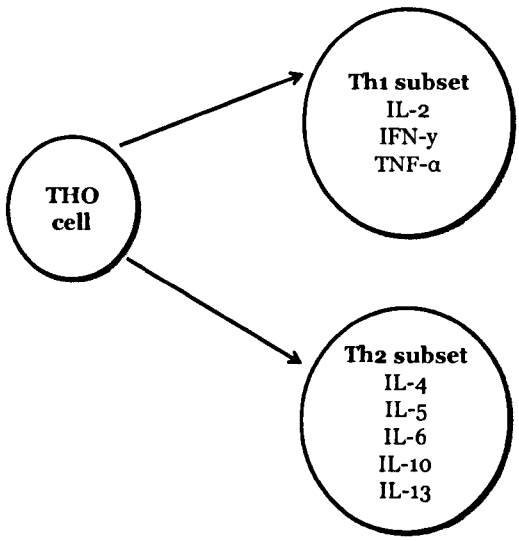

Figure 7: Th1 and Th2 differentiation subsets

(Adapted from Opal & Depalo 2000)

Evidence for a Th2-type profile in RAO includes elevated concentrations of mRNA for IL-4, IL-5 and IL-13 from BAL fluid RAO-affected horses, with decreased expression of IFN-γ (Lavoie *et al* 2001, Beadle *et al* 2002, and Cordeau *et al* 2004). Additionally, a 'modified Type 2 cytokine response', has been described where mRNA for IL-4, IL-13 but not IL-5 was expressed in RAO-affected horses (Horohov *et al* 2005). Some studies have not established Th1 or Th2 type cytokine responses in RAO-affected horses (Kleiber *et al* 2005, Pietra *et al* 2007); others found increased concentrations of IFN-γ indicating a Th1 influence for RAO pathogenesis (Ainsworth *et al* 2003). The lack of conclusive evidence for a Th2 based response suggests that RAO may not be an exclusively allergen-driven condition.

Non-allergic or refractory asthma

Conflicting results in establishing a definite Th2 phenotype in RAO could be attributed to

- differences in sampling methods and measurements
- varying states of disease progression/ antigen exposure times
- The timing of sample collection.

Other variations include the age and type of horses used in studies, and type of allergen challenge (older studies frequently only cite 'dusty' bedding or hay). Furthermore, although cytokine profiles may be skewed towards Th2 or Th1 subsets, both types continue to produce their respective subset cytokines to some degree (Horohov *et al* 2005).

Investigations into the differing forms of asthma in humans suggest a late-onset or 'intrinsic' phenotype in adult life unrelated to atopic sensitization. Production of Th2 cytokines and IgE has been demonstrated in non-allergic asthma (Wenzel 2001). Contributory factors to intrinsic asthma include: sensitization to environmental factors unconnected to IgE influence, and airway reactivity to prolonged tissue damage (Holgate 2008). If a similar intrinsic phenotype occurs in horses with RAO, it has not yet been established.

1.12 Respiratory inflammation and airway remodelling in RAO and asthma

Histology and cell types of the trachea

Gas exchange quality and respiration rate are reliant on healthy, structurally intact airway tissue. Air delivery to the lungs in asthma and RAO is restricted by smooth muscle contraction of the bronchi and bronchioles, inflammation of the bronchial mucosa and excess mucus in the airways. The mucosal layer lining the lumen of the trachea (**Fig.8**) and

primary bronchi is composed of ciliated pseudostratified columnar epithelium and the lamina propria (Beachey 1998).

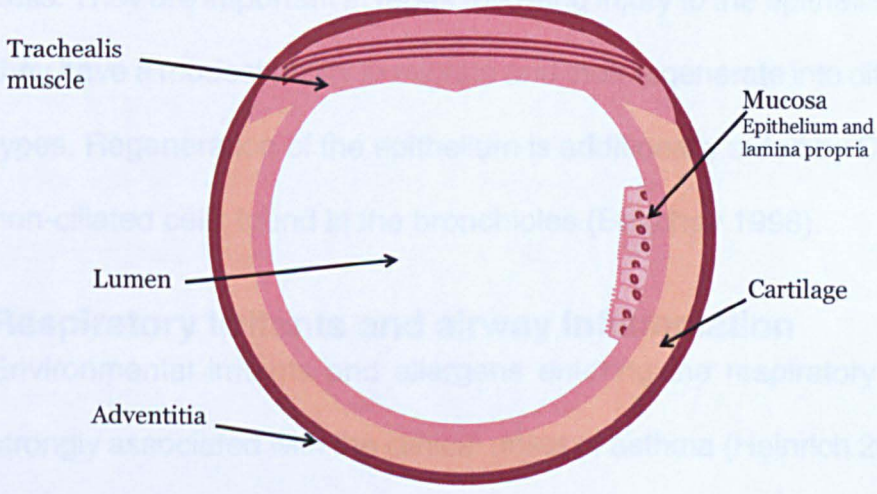

Figure 8: Cross section of the main tissue types found in the trachea
(Figure adapted from Beachey 1998).

The predominant cell types in the respiratory epithelium are ciliated pseudostratified columnar cells, goblet (mucus) cells and basal cells (Beachey 1998). Mucosal dendritic cells (DCs) and Clara cells are also present in the epithelial layer (Iwasaki 2007).

The ciliated pseudostratified columnar cells form a single layer in contact with the basement membrane. Visually, they appear to be in many layers or 'pseudostratified'. These cells are secured at their apical, lateral and basal surfaces to effectively form an epithelial barrier.

Goblet cells are cup-shaped cells in the lower respiratory tract (LRT) whose numbers decrease towards the bronchioles. They are simple columnar cells with membrane-bound mucin granules which are secreted and used in the formation of mucus.

Basal cells are small, roughly triangular shaped cells. They aid attachment of epithelial cells to the basal layer and are a reserve pool for epithelial cells. They are important in repair following injury to the epithelial layer, as they have a modest ability to migrate and then regenerate into different cell types. Regeneration of the epithelium is additionally aided by Clara cells, non-ciliated cells found in the bronchioles (Beachey 1998).

Respiratory irritants and airway inflammation

Environmental irritants and allergens entering the respiratory tract are strongly associated with the clinical onset of asthma (Heinrich 2011). The aetiopathogenesis of RAO indicates that inhalation of environmental stable dust is the main instigator of RAO (Gerber *et al* 2008).

Horses in the UK are frequently stabled for extended periods, particularly in winter (Hotchkiss *et al* 2007). There is a connection between poorly ventilated stable environments and airway inflammation in susceptible horses (Clements & Pirie 2007, Marinkovic *et al* 2007). Subclinical airway inflammation and mucus accumulation may occur in horses in the absence of clinical signs. These findings are correlated with being housed in a conventional stable environment (Gerber *et al* 2003), which offers limited ventilation.

Stabling increases exposure to organic dust, abundant in poor quality feed or bedding. Organic dust which enters the peripheral airways exacerbates RAO (Robinson *et al* 1996, Clements & Pirie 2007). RAO has been described as being similar to occupational or organic-dust induced asthma in humans (DeLuca *et al* 2008). Grain dust exposure is shown to increase pro-inflammatory cytokine IL-6 expression in the human airways (Deetz *et*

al 1997, Von Essen *at al* 1998). This theory is further supported as clinical remission of RAO can be achieved if the animal is placed in a low dust, well ventilated environment (Dacre *et al* 2007).

Other inhaled agents capable of inducing airway inflammation include noxious gases, moulds, and bacterial cell wall toxins (endotoxin) from Gram–negative bacteria or their lipopolysaccharide (LPS) derivatives. Moulds contributing to RAO pathogenesis include (1→3)-β-D-glucan from thermophilic types *Aspergillus fumigatus*, *Thermoactinomyces vulgaris,* and *Faenia rectivirgula* (Pirie *et al* 2003).

Exposure to extracts from these moulds causes pulmonary inflammation in RAO-affected horses (McGorum *et al* 1993). However, the extent of mould-induced respiratory inflammation is less when compared to that initiated by hay or straw exposure. The potency of moulds considered to initiate RAO exacerbations is thought to be enhanced by airborne LPS fractions (Pirie *et al* 2003).

Effects of airborne and systemic endotoxin in horses and humans

The concentration of airborne endotoxin is increased 8-fold in the environment of stabled horses, compared to those at pasture. Endotoxin, in very small concentrations, is noted for provoking a rapid innate immune response, by activating Toll-like receptors and initiating inflammation. The effects of endotoxin are credited to its highly potent lipid-A moiety (Liu *et al* 2002).

Endotoxin exposure also recruits neutrophils to the airways, a typical feature of RAO (Pirie *et al* 2001). Inhalation of 1.6 and 5 mg *A. fumigatus*

extract increased neutrophil numbers and lung dysfunction in RAO-affected horses. Removal of LPS fractions from *A. fumigatus* extract attenuated the increase in neutrophil numbers (Pirie *et al* 2003).

Clinical manifestations of endotoxemia in human patients include: muscle tremors, lowered blood pressure, tachycardia, and tachypnoea. Multiple organ failure and death may occur in severe untreated cases (Pleiner *et al* 2002). Additional signs of endotoxemia in horses include vascular dysfunction, systemic inflammation, and pulmonary hypertension.

Horses are sensitive to the effects of LPS exposure; like human patients, endotoxemia in horses is viewed as a significant cause of mortality (Morris 1991, Moore 2001). Equine mortality rates from gastro-intestinal dysfunction or systemic inflammation are increased by the effects of endotoxemia (Neuder *et al* 2009).

The relative contribution of either organic dust or LPS fractions to the induction of airway inflammation in RAO is undefined. However, LPS is a highly potent agonist for the human and equine immune system (Moore 2001). LPS contributes significantly to development of acute and chronic airway inflammation in many human and equine diseases. It can therefore be regarded as an important factor in respiratory disease.

Airway remodeling in RAO and asthma

Episodes of inflammatory exacerbation in asthma and RAO can cause significant tissue damage. The presence of a continued pulmonary insult instigates airway hyper-responsiveness (Berend *et al* 2008) in a self-perpetuating cycle of respiratory injury (**Fig.9**).

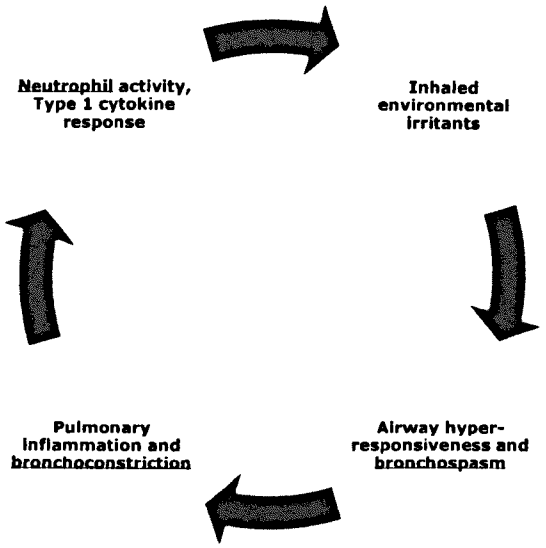

Figure 9: Cycle of airway damage and injury in RAO and asthma

(Adapted from Beachey 1998)

Over time, repeated inflammatory episodes from exposure to environmental pathogens will cause alterations to the surrounding respiratory tissue. Such alterations may inhibit or affect the healthy functioning of the lung and are classed as airway remodelling (Marinkovic *et al* 2007, Berend *et al* 2008).

Dysfunctional changes to epithelial cells from repeated inflammation

The pulmonary mucosa is an integral part of innate and adaptive immunity. It represents the first point of contact for host defence against airborne pathogens and actively modulates airway function (Knight & Holgate 2003, Kato & Schleimer 2007).

The effect of unresolved pulmonary inflammation is noted as a factor in the pathogenesis of human chronic lung conditions (Polito & Proud 1998). Allergen-induced inflammation can create crosstalk between mucosal DCs and epithelial cells. This crosstalk has been correlated with the initiation of asthma (Hammad & Lambrecht 2008).

Persistent or prolonged inflammation causes loss of epithelial integrity and the physical defence it provides (Matera *et al* 2002, Kato & Schleimer 2007). Injury to the protective mucosal layer in equine gastro-intestinal conditions enhances the damaging effect of endotoxin (Moore *et al* 2001). Damaged respiratory epithelial cells become increasingly permeable to airborne pathogens or environmental pollutants (Polito *et al* 1998, Greene & McElvaney 2005, Knight & Holgate 2003, Mayer *et al* 2008, and Holgate 2008).

Signs of uneven epithelial repair and structural damage are seen in RAO-affected horses (Berend *et al* 2008). Airway remodelling may develop following prolonged respiratory damage and is evident through tracheal changes. This includes: epithelial thickening, goblet cell hyperplasia, basement membrane thickening, increased cellular matrix and hypertrophy or hyperplasia of ASM (Robinson 2001). Tracheal changes contribute to significant airway wall narrowing with diminished airflow in RAO and asthma. AHR significantly increases bronchoconstriction and irreversible lung damage in both conditions (An *et al* 2007). The ability of the pulmonary mucosa to repair may subsequently deteriorate (Yang *et al* 2006).

The airway epithelium regulates airway function, has significant roles in immunity, and directly affects the development of airway remodelling. This suggests that the epithelial layer is pivotal in the modulation of airway disease. Minimising pulmonary inflammation and epithelial damage would therefore seem to be fundamental in the treatment or inhibition of RAO.

Increase in smooth muscle layer in RAO and asthma

An increase in the thickness of the smooth muscle layer surrounding the airways is a feature of both RAO and asthma (Hirst *et al* 2004, Herszberg *et al* 2006). ASM thickening can significantly restrict airflow during episodes of bronchoconstriction (Bai and Knight 2005). The severity of both conditions is thought to be reflected by the advancement of ASM increase (Robinson 2001, James *et al* 2012).

Targeting the pathogenesis of ASM increase is a focus of asthma studies and RAO investigations. ASM increase in humans is attributed to muscle cell hyperplasia, hypertrophy and a reduction or failure in cell apoptosis (Hirst *et al* 2004). ASM hyperplasia found in small and large airways of asthmatics is particularly noted as a major factor in fatal asthma (James *et al* 2012). A similar scenario is evident in horses with RAO where ASM proliferation is attributed to both increased myocyte proliferation and apoptosis. It is suggested that the abnormal apoptosis serves as a way to limit the increase in ASM growth (Herszberg *et al* 2006).

The relative contribution of either hyperplasia or hypertrophy towards airway remodelling in asthma remains under debate, and is largely un-investigated in RAO. Typical mitogens and inflammatory factors cited as capable of ASM increase in asthma include epidermal growth factor, platelet-derived growth factor isoforms, fibroblast growth factor 2, tryptase, endothelin-1, thromboxane A2 leukotriene D, IL-1β, IL-6, and TNF-α (Hirst *et al* 2004). The source of these factors originates from crosstalk between the damaged epithelium and ASM or the innate immune response of ASM cells (James 2005). Epithelial loss and smooth muscle

hyperplasia have been recorded in children with asthma, suggesting the strong relationship between alterations in the epithelium and abnormal ASM thickening (Zhou *et al* 2011). Continued expression of inflammatory mediators from damaged epithelium may promote ASM hyperplasia (James 2005).

Vascular smooth muscle cells are noted as having the capacity to change their phenotype in response to the localized environment (Alexander and Owens 2012). A similar ability may exist in equine and human smooth muscle cells. Smooth muscle phenotype in asthma is proposed as one potential mechanism for the transformation of healthy cells into asthma-like cells, which may have differing capacities to generate markers and inflammatory mediators. Conventional asthma treatment fails to fully control the extent of smooth muscle alteration (An *et al* 2007).

Evaluation of airway inflammation and remodeling

In order to investigate the mechanisms of inflammation and remodeling in RAO and asthma, numerous methods have been developed *in vitro* in various spices.

Equine and human models of the airway epithelium *in vitro*

In vitro cell culture models are a vital tool in the study of airway diseases mechanisms in horses and people. They have made significant contributions to understanding inflammatory disturbances, bronchial tissue alterations and the efficacy of new drugs for clinical research.

In vitro pulmonary models provide several advantages over studying respiratory disease *in vivo* or *ex vivo*. *In vitro* methods afford the ability to

carefully control the culture environment, exclude variability in data from other respiratory cell types, and study primary cell characteristics such as differentiation, repair potential and specific responses to pathogens.

Equine and human models of the airway epithelium *in vitro*

In vitro systems include 2-dimensional monolayer cell systems with continuous or primary cells, and 3-dimensional studies composed of air-liquid interface and organotypic cultures.

Two-dimensional cell culture studies

Human continuous respiratory cell lines

Continuous respiratory cell lines are derived from human lung carcinomas, or those created by mutagenesis. Continuous cell lines can provide valuable information when investigating human respiratory models of disease. Advantages include increased availability over primary cell cultures, and consistency between cell batches whereas primary cells may vary. Assessment of the physiological aspects and disease mechanisms of epithelial cells are often studied using an immortalized cell line BEAS-2B, a continuous cell line derived from lung tissue and demonstrating epithelial cell morphology.

Primary bronchial cell culture

Primary bronchial epithelial cells have been successfully isolated and cultured from human, bovine, canine and murine species (Spurzem *et al* 2005, Kato *et al* 2006, Newby *et al* 2007, Erles & Browlie 2010). Initial methods to culture primary equine bronchial cells have been described by incubation of equine tracheal segments for 24hrs in media containing 0.1% pronase (Sime *et al* 1997). Similar works have isolated respiratory

epithelial cells by digesting equine nasal tubinates in a Type I collagenase/type XIV protease mix (Lin *et al* 2001), and by 0.1% protease digestion of isolated equine bronchi (Ainsworth *et al* 2009). These studies required serum supplementation of cell media and use of collagen coated culture flasks to aid initial growth and cell adherence.

High yields of viable ETE cells have been obtained by digesting tracheal mucosal epithelium in media containing 0.25% trypsin. In contrast to primary cells isolated with pronase/protease, the use of collagen-coated culture flasks was unnecessary after initial dissociation of cells from mucosal tissue (Shibeshi *et al* 2008). Additionally, cellular characterisation is described in this study after primary cells were maintained for extensive periods in culture. Monolayer cells developed beyond cobblestone morphology and were described by the authors as 'pseudostratified columnar epithelium-like'.

Culture of primary airway smooth muscle cells

Primary airway smooth muscle cell culture has also been established in humans, rabbit, cow, and dog, although relatively unstudied in the horse (Hirst *et al* 1995, Bonnaci *et al* 2003, Halayako *et al* 2005, Alkhouri *et al* 2011). Initial work by Fleischmann *et al* (1993) describes the enzymatic dissociation of primary myocytes from ferret trachealis muscle using Collagenase D and elastase. This method was further developed for collection of equine myocytes by digestion of minced trachealis muscle for 20 mins in collagenase (Fleischmann *et al* 1996). Additional work with this method determined electrical activity but did not detail culture or identification of isolated cells.

Equine culture of primary smooth muscle cells has primarily focused on vascular smooth muscle. Smooth muscle cells have been successfully cultured from tunica media explants derived from equine palmar digital arteries (Rodgerson *et al* 2001). Vascular smooth muscle cells derived from explants of ascending aorta, pulmonary artery and digital artery are also reported (Janicke *et al* 2003). Cells were also maintained in serum-enhanced media and identified by ICC staining with anti-α-actin monoclonal antibody (clone 1A4). Neither study details the length of time taken for primary cells to become confluent, or attempts to passage cultured cells.

Three-dimensional studies of asthma and RAO

Two dimensional lung models are often criticised as not being physiologically realistic. Accordingly, increasingly sophisticated representations of the lung are being developed to enable exploration of morphological and immunological mechanisms of disease (Pampaloni *et al* 2007). Relevant model systems for equine three dimensional respiratory cell studies include air-liquid interface systems (ALI), respiratory explants and organ tissue culture (Pampaloni *et al* 2007).

ALI cell culture

Bronchial cells grown at the air-liquid interface are regarded as a significant contribution to investigations of respiratory biology (Keismer *et al* 2009). Immortalized human bronchial epithelial cells exhibited the capacity to differentiate when cultured in a three-dimensional system (Delgado *et al* 2011). BEAS-2B cells maintained at the air-liquid interface (ALI) developed into basal, mucin-producing, and columnar ciliated epithelial cells (Vaughan *et al* 2006). Similarly, primary equine bronchial

epithelial cells differentiated when cultured at the ALI and demonstrated the capacity for mucus production (Schwab *et al* 2010), cilliogenesis and tight junction formation (Quintana *et al* 2011).

Organ ALI culture

Cultures of equine mucosal explants *in vitro* have been described by Vandekerckhove *et al* (2009). Mucosal tissue explants from vestibular, intranasal septum, the nasopharynx and the trachea were maintained in serum free media at the ALI for up to 96hrs without loss of morphology or epithelial integrity. Explants of equine mucosal tissue maintained on fine mesh gauze for up to 96 hrs demonstrated EHV-1/EHV-4 disease mechanisms highly similar to *in vivo* reactions (Vanderhocke *et al* 2011).

Whole organ explant cultures are also described in the horse (Lin *et al* 2001), where tracheal rings were dissected into sections with cartilage and basement membrane intact. Tracheal sections were maintained epithelial side up on a gyrorotary platform in a CO_2 free atmosphere. Explants maintained in these conditions were viable for 48hrs before morphological degeneration of the epithelium became evident.

1.13 Summary

RAO and asthma are complex respiratory diseases where repeated inflammation instigates airway hyperesponsiveness and subsequent alteration of bronchial tissue. Lack of LXA4 and receptor ALX in severe asthma suffers is a potential mechanism of the development of chronic inflammation. Use of an *in vitro* equine respiratory model with selected mediators (**Table 5**) to examine LPS induced inflammation and potential

inflammatory resolution by LXA4 may aid understanding of the pathogenesis of RAO and asthma.

Mediator/ molecule	Classification	Principal action
ALX	receptor	Initiation of anti-inflammatory pathways
TLR4	receptor	Initiation of inflammatory pathways
TNF-α	cytokine	Inflammation
IL-1β	cytokine	Inflammation
iNOS	enzyme	Inflammation
COX-2	enzyme	Inflammation/inflammatory resolution

Table 5: Important mediators in RAO and asthma

1.14 Thesis hypothesis

Equine inflammation and its resolution can be modelled *in vitro* using respiratory epithelial and smooth muscle models.

1.15 Aims of the thesis

- Asses and select in vitro equine models of respiratory epithelium and airway smooth muscle
- Characterise the response of selected models to LPS, a known instigator of inflammation on chosen mediators of inflammation and inflammatory resolution.
- Compare the response of selected models to LXA4, considered to initiate inflammatory resolution, on chosen mediators of inflammation and inflammatory resolution.

Chapter 2: Materials and Methods

Details are given here of standard methods used in the chapters describing inflammation and inflammatory resolution (Chapters 4, 5 and 6) in the *in vitro* respiratory model. Specific details for any methods used are described in each individual chapter.

2.1 Materials

Cell culture grade PBS, F-12 (Hams) Nutrient mixture, 10,000U/ml penicillin/10,000µg/ml streptomycin (Pen/Strep), 250µg/ml Fungizone (Amphotericin B in water), SYBR Green master mix, Alex-Fluor 488, all NuPage reagents/gels and MagicMarkTM XP, were purchased from Invitrogen, Paisley, UK.

PVDF membranes were obtained from Amersham, Buckinghamshire. BEGM was obtained from Lonza; Slough, UK with porcine trypsin/2.65mM EDTA, heat inactivated FBS, Leibovitz's 15 and DMEM from Fisher Scientific, Loughborough, UK. Polysciences Polybead Microsphere® beads and 13mm tissue culture cover-slips-were acquired from Sarstedt, Leicester, UK.

Pan-cytokeratin PCK-26 was sourced from AbCam, Cambridge, UK and antibody smooth muscle actin (clone 1A4) was acquired from Dako, Cambridgeshire, UK as was Expose Polyvalent HRP/DAB Detection kit.

Antibodies for ALX and COX-2 for Western Blot were obtained from Novus Biologicals, Cambridge UK, and New England Biolabs, Ipswich, UK

respectively. Antibody for β-actin was a kind gift from Dr S.Dunham (Nottingham School of Veterinary Medicine and Science, Nottingham).

The Bradford assay reagent was purchased from Bio-Rad, Hemel Hempstead, UK and RIPA buffer obtained from Sigma-Aldrich, Poole, UK. LipoxinA4 and the nitrite/nitrate fluorometric assay kit were supplied by Cayman Chemicals, Cambridge Biosciences UK.

The full address of each supplier is detailed in appendices

2.2 Preparation of cultured equine primary cells and treatment protocols

Preparation of equine primary cells prior to incubation with vehicle
Culture media for primary equine cells was removed and replaced with fresh culture media 12 hrs prior to treatment. All cell populations were washed with warmed sterile cell-culture grade PBS (Invitrogen, Paisley, UK) which was removed and discarded before incubation with the relevant vehicle.

Incubation of primary cells with lipolysaccharide, carprofen or lipoxin A4
Cells were incubated with fresh medium (control) or medium containing LPS from *Escherichia coli* (serotype 0127:B8), (Sigma-Aldrich), carprofen and LXA4, (Cayman Chemicals, Cambridge, UK). Concentrations of each treatment are indicated (**Table 6)** with specific amounts and incubation times detailed in the relevant chapters.

Treatment	Concentration per ml
Lipolysaccharide	0.1- 100µg
Carprofen	4 µg
LXA4	100µM

Table 6: Primary cell treatment types and concentrations

Preparation of treatment vehicle solutions

A stock solution of 1mg/ml LPS solution was prepared under sterile conditions by dissolving *E.Coli* powder (Sigma-Aldrich, Poole, UK) ground with 25mls of sterile cell culture media, aliquoted and kept at -20°C.

Carprofen solutions were made from 2mg of Rimadyl® tablets (Sigma-Aldrich, Poole, UK) ground in a sterile mortar and pestle, added to 50mls of cell-culture media and sterile filtered using a 0.2µM syringe filter (Whatman, Maidstone, UK). Carprofen solutions were used immediately.

A 50µl aliquot of LXA4 (25µg of product in 250 µl of solution) was evaporated under a nitrogen stream for 20 minutes. A 5µg/ml stock solution was prepared by adding 1ml of sterile cell culture grade PBS.

2.3 Isolation of ribonucleic acid (RNA)

Total RNA from cell samples was extracted using commercial RNA purification kits (RNeasy Mini kit, Qiagen and Pure Link, Invitrogen, Paisley, UK). All plastic-ware and materials were certified RNAse free by the manufacturers, with additional treatment for 45 minutes in a ultra-violet flow hood. Cell samples (approximately 20µg of tissue or 4.8×10^5 cells) were lysed with a 600 µl volume of guanidine-isothiocyanate lysis buffer

with 10 µl of β-mercaptoethanol added. The cell lysate was then passed through a disposable sterile 21-gauge needle and sterile plastic 1ml syringe 4-5 times. Total homogenisation was achieved by micro-centrifugation of the cell lysate in spin columns supplied in the kit.

RNA extraction and elution was performed using silica-gel membrane purification according to the kits instructions. Additionally, an on-column digestion of DNA (RNase-Free DNase, Qiagen) in each sample was performed. Each sample was incubated with 80 µl DNase mix for 15 minutes at room temperature to eliminate contaminating genomic DNA.

The RNA quality and concentration of each sample was assessed through spectophotometric absorption according to Beer-Lambert law. RNA sample concentrations ranged from 0.10-1 µg of total RNA. Samples with an absorption ratio of >1.7 were considered to be sufficiently pure for use in first strand complementary deoxyribonucleic acid (cDNA) synthesis. Quantified RNA samples were aliquoted into 10µl samples for -80°C storage or used immediately in cDNA synthesis.

2.4 Reverse transcription of total RNA for synthesis of first strand cDNA

Samples of cDNA were obtained from isolated RNA by use of the SuperScript III First Strand Synthesis System (Invitrogen, Paisley, UK). All reagents for total cDNA synthesis were supplied by the manufacturer. All samples were standardized to ensure that equal concentrations of RNA per sample were used in cDNA synthesis.

Total RNA samples were reverse-transcribed into cDNA by the use of either oligo-dT or random hexamers in three main stages. The initial step and synthesis mix (**Table 7**) enabled the linearization of RNA secondary structures.

Component	Volume
0.10-1 µg total RNA	*N*µl
50 ng/µl random hexamers	1 µl
10 mM dNTP mix	1 µl
DEPC-treated water	*N*µl
Total volume	10µl.

Table 7: Components of initial stage of cDNA synthesis mix

Samples were incubated for 65°C for 5 min and placed on ice for 1 min. The second step enabled annealing of the random hexamers to the RNA and subsequent formation of cDNA. Each sample had 10 µl of second stage cDNA synthesis mix added (**Table 8**).

Component	Volume per sample reaction
10X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNaseOUT (40 U/µl)	1 µl
SuperScript III RT (200 U/µl)	1 µl
Total volume	20 µl

Table 8: Components of the second stage of cDNA synthesis mix.

Each sample reaction was then incubated for 10 minutes at 25°C followed by 50 minutes at 50°C. Reactions were then terminated by heating samples at 85°C for 5 min. All samples were immediately chilled on ice.

To complete the cDNA synthesis each sample had 1 µl of RNase H added before a final incubation step for 20 min at 37°C. All samples were used directly in polymerase chain reactions (PCR) or stored directly at -80°C.

2.5 End-point and real time polymerase chain reaction (PCR)

End-point PCR

End-point and real time PCR (q-PCR) methods were both used to amplify specific genes of interest. This was achieved by either separation of PCR products through agarose gel electrophoresis (end-point) or detection of PCR product by emission of a fluorescent signal (real-time).

PCR primers and primer design

The primers sequences and conditions used in end-point and q-PCR were obtained from either published papers (Berndt *et al* 2007, Zhang *et al* 2009), from Dr Richard Hammond (personal communication), Dr Steve Dunham (personal communication) or designed using NCBI-BLAST primer design.

To minimise non-specific annealing (primer-dimers), primer pairs used in experiments (**Table 9**) were designed to be 18-22 bases long, with a GC content of no more than 50%. Annealing temperatures for each sense and anti-sense strand were 60°C or with no more than 1°C difference between the primer pairs. Primer pairs and their predicted amplicon length for each gene of interest were verified via NCBI-BLAST and EMBL-EBI multiple sequence alignment programme ClustalW2.

Gene	Amplicon size	5'-3'strand (sense)	3'-5'strand (antisense)
ALX	213	AAACCACCGCACTGTAGGTC	CGGATGATCCCTCTGACTGT
TLR-4	91	TCTGGAGACGACTCAGGAAA GC	GCAAGAAGCACCTCAGGAGTTT
TNF-α	74	CGACTTTGCGGAGTCCGGGC	GTGGGCGAGGACGGACGTAC
IL-1β	69	CTGCAGCGGCAATGAGAAT	TGGAAGCTGCCCTTTCATCTG
iNOS	372	TAGAGGAACATCTGGCCAGG	TGGCAGGGTCCCCTCTGATG
COX-2	320	GAGTACCGCAAACGCTT	TTATTGCAGATGAGAGACTG
β-actin	107	CACCACACCTTCTACAAC	ATCTGGGTCATCTTCTCG
GAPDH	365	GAGATGATGACCCTTTTGGC	GTGAAGGTCGGAGTCAACG
18-S	204	ATGCGGCGGCGTTATTCC	GCTATCAATCTGTCAATCCTGTCC

Table 9: PCR primer pairs used in experimental studies

End-point PCR

A standard 25 µl PCR reaction mix (**Table 10**) was prepared in a 0.2µl sterile disposable RNA/DNA-free eppendorf.

Component	Volume
GoTaq Hotstart Colourless mix	12.5µl
Sense primer	1.5µl
Anti-sense primer	1.5µl
DEPC –treated water	8.5µl
Sample of cDNA	1µl
Total volume	25 µl

Table 10: End-point PCR synthesis mix.

All reactions were kept chilled on ice. Reference genes GAPDH, 18S or Beta-actin were used to measure constitutive cellular RNA expression and no template controls (NTC) omitted cDNA to act as negative controls.

Samples were placed in an automated thermal cycler with a heated lid and run using a standard PCR protocol. An initial 2min denaturing step of a single cycle at 95°C was followed by 35-45 consecutive cycles of three distinct stages (**Table 11**). A final extension step was performed at 72°C for 10 minutes to allow total synthesis of full length products.

PCR cycle	Cycle temperature	Cycle time
Denaturing	95°C	60 seconds
Primer annealing	50–65°C	60 seconds
Extension/elongation	72°C	60 seconds

Table 11: End-point PCR protocol for cycle temperature and time

Annealing temperatures (Tm) for each primer pair were used where stated in the original papers. If required, Tm was optimized thereafter, by calculated by the percentage of G and C bases in the primer.

Agarose gel electrophoresis

The predicted size of the PCR products determined the % (w/v) of agarose used in the gel. DNA products of <500bp were separated on gels of 2%, 1.5% gels for products of 500bp -1200bp and 0.8% for products of >1200bp. Agarose gels were run for approximately 90 minutes at 100V. Agarose powder and 1 x Tris-EDTA acid buffer (TAE, 40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA, pH 7.6) were heated until the agarose had

fully melted. The solution was cooled under cold running water to approximately 37°C.

Ethidium bromide (3.5µl) was mixed in with the agarose solution and the gel poured into a casting tray. The gel was placed into a PCR tank, covered with 1 x TAE buffer and samples loaded. DNA loading buffer was used to prepare the samples at a ratio of 3µl buffer to 10µl cDNA sample (1:3 ratio), with 12µl of buffered sample loaded into each well. A standard 1 Kb DNA ladder (10 µl) was separated with the samples and used to estimate the size of PCR products.

Sequencing of PCR products

Products from endpoint PCR were used to determine that the chosen primer pairs were successfully amplifying the relevant gene of interest. PCR samples were sent for commercial Sanger DNA sequencing to SourceBioscienes, Nottingham. A typical 6µl PCR sample for sequencing contained a cDNA concentration of 1ng/µl per 100bp. The generated amplicon sequences were verified with NCBI-BLAST, and are detailed in the appendices.

Real time PCR

The relative concentration of RNA in samples was quantified by emission of a fluorescent signal by use of qPCR. SYBR Green (Invitrogen, Paisley, UK), a fluorogenic dye that binds to double stranded DNA, was used to detect the amount of reverse-transcribed cDNA in samples. The strength of signal emission is proportional to the copy number of the gene of interest.

Real time PCR synthesis mix and cycling programmes
Each cDNA sample was diluted 1/25 in DEPC-treated water before the addition of a SYBR Green (Invitrogen, Paisley, UK) master mix (**Table 12**).

Component	Volume per reaction
SYBR Green	10µl
DEPC-treated water	2.2 µl
Primer	2.8 µl (1.4 of each primer)
cDNA or DEPC-treated water for NT control	5 µl
Total volume	20 µl

Table 12: Components of real time PCR SYBR Green master mix.

Each individual reaction was repeated in triplicate, with all samples loaded into a 96-well plate. Negative controls were represented by the omission of cDNA in samples to act as a no template (NT) reaction.

Samples were run on a Lightcycler 480 (Roche, West Sussex UK) with thermal cycler conditions as follows: incubation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 10s, 60°C for 15 and 72°C for 30s. A final extension step of 10 minutes at 72°C was performed.

Standard curves and relative quantification of product for q-PCR
Standard curves were used to measure the concentration of product and the efficiency of all primer pairs. Standard curves were constructed from a serial dilution of pooled equine tracheal epithelial cells, airway smooth muscle cells (Chapter 3), and equine mononuclear cells. Dilution factors were 5-fold, with each reaction in duplicate and as follows: 1:5, 1:10, 1:250, 1:1250, 1:6250 and 1:31250.

The efficiency of each primer pair used was then calculated from the anti-log concentration generated from Ct values and each serial dilution. A correlation coefficient of 1.000 indicates a perfect fit for the standard curve.

Quantification of mRNA samples in real-time PCR

Values for the absolute concentration of each mRNA sample were derived from the mean average of each triplicate reaction. A melt curve analysis on the final product indicated whether a specific product was amplified and the presence of primer-dimers. Relative quantification of the mRNA samples was then calculated by dividing target gene values with reference gene values.

Isolation of peripheral blood mononuclear cells from whole blood for real time PCR standards

Peripheral blood mononuclear cells from whole blood were used to provide a positive control for ALX real time PCR standards. Whole blood samples (10mls) were obtained from Welsh Mountain ponies by jugular venipuncture into vacutainers and kindly provided by Dr Julia Kydd (School of Veterinary Medicine and Science, Nottingham).

All samples were collected with 150 units/ml of heparin to prevent coagulation of blood. Peripheral blood mononuclear cells (PBMCs) were isolated using a standard Ficoll-Paque gradient method within 2 hours of collection. The plasma layer (10mls) was removed from each tube and carefully layered over 4.5mls of Ficoll-Paque (GE Healthcare, Chalfont St Giles, UK) in a 15ml sterile disposable polypropylene tube. Samples were centrifuged at 400 x g for 10 minutes at 20°C. The upper layer of supernatant was aspirated, and the layer containing PBMCs transferred

into a fresh 15ml sterile tube. Cells were washed twice in 5mls sterile PBS followed by centrifugation at 400 x g for 10 min at 20°C to remove platelets. The cell pellet was re-suspended in D-MEM (high glucose) with 10 % FBS (Invitrogen, Paisley, UK).

Statistical Analysis of q-PCR values
Values for mRNA expression were derived as mean numbers from triplicate samples following normalization to the reference gene from each individual experiment. Statistical significance was determined through STDEV, standard SEM and ANOVA analysis of the raw data. A *p*-value of <0.05 in ANOVA analysis was regarded as statistically significant.

2.6 Western blot

Preparation and quantification of protein samples
Cultured primary equine cells were grown to 70-80% confluency as described. An ice-cold RIPA lysis/extraction buffer containing phosphatase and protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail), (Roche, West Sussex UK) was used to harvest cells. Cell samples were kept on ice and incubated for 30 mins in 100µl per sample of prepared RIPA buffer. Cell lysates were then centrifuged at 800g, 4°C for 10 minutes. The resulting cell supernatants were stored at -80°C before use in protein studies.

Quantification of protein samples using the Bradford protein assay
The concentration of protein in each sample was ascertained by the use of the Bradford protein assay (BioRad). This assay employs colorimetric absorbance of Coomassie Blue dye with protein concentration dependant on the amino acids present in the sample. Sample readings are taken at 595 nm where the absorption spectrum of the dye is held.

Protein standards and quantification of samples
A set of 5 protein standards (0.05mg/ml to 0.5 mg/ml) to quantify unknown samples were prepared by diluting 1.45mg/mL BSA in double distilled water. A 1:4 dilution of Bradford reagent (BioRad) was prepared with sterile water and filtered through filter paper. A 10 µL sample of each protein standard or unknown was mixed thoroughly with 200µL of the diluted Bradford reagent. All standards and samples were repeated in triplicate in a 96 well plate. Samples were incubated at room temperature for 10 minutes, then protein concentrations quantified with the Fluorostar microplate reader set at 595 nm

Denaturing of protein samples for separation on polyacrylamide gels
Protein samples were denatured prior to separation through electrophoresis on polyacrylamide gels. Up to 7.5µl of protein sample containing 25µg/ml of protein was combined with 2.5µl of NuPage LDS. Sample buffer (4x) and 1 µl of NuPage Reducing Agent (Invitrogen, Paisley, UK), to make a total volume of 10 µl. Samples were then heated in a thermal block at 70°C for 10 minutes. Denatured samples were used directly in electrophoresis or stored at -80°C.

Separation of proteins from cell lysates on polyacrylamide gels.
Prepared denatured proteins were separated on a precast 8-12% NuPage Bis-Tris gel used in a NuPage X Cell Sure Lock module (Invitrogen, Paisley, UK). NuPage SDS Running Buffer (MES, 20 xs) was prepared by adding 50mls of stock solution to 950mls of chilled deionised water and used to fill the module. The inner module chambers then had 500µl of NuPage antioxidant added to ensure optimal separation of proteins. The

denatured samples and protein ladder were loaded at 10µl per sample per well. Gels were then run at approximately 200V for 1 hour.

Protein transfer onto PVDF membrane
Separated proteins were transferred from polyacrylamide gels after electrophoresis onto PVDF membranes (Hybond-P), (Amersham, Buckinghamshire, UK). Transfer buffer was prepared by combining 50mls of NuPage Transfer buffer, 1ml of NuPage antioxidant, and 100ml of methanol per gel made to a final 1L volume with deionised water. The PVDF membrane was soaked in 100% methanol for 30s prior to use. Proteins were transferred in an XCell II Blot module filled with prepared buffer for 1 hr at 30V.

Detection of transferred proteins and visualisation using ECL
Three main steps were performed before ECL detection: blocking the membrane to prevent background staining, overnight incubation with the primary antibody, and then subsequent incubation with the secondary antibody

Blocking of PVDF membranes
The PVDF membranes were blocked for 1 hr at room temperature in 200mls of 5% BSA solution made with 0.2% TBS-Tween. The membrane was then washed 3-4 times with TBS-Tween before incubation with the primary antibody.

Incubation with primary antibody
Membranes were then incubated for 12-14 hrs at 4°C in 10mls of primary antibody added to a 5% BSA solution (diluted in 0.2% PBS-Tween). **Table 13** gives the details, concentrations and predicted molecular weight of the antigens recognised by the primary antibodies. The concentration of

primary antibodies was obtained according to manufacturer's recommendations.

Primary Antibody	Concentration	Species primary antibody was raised in	Molecular weight (kDa)
ALX Target antigen is FPR 1/2 (ALX receptor) in human	1/500	Mouse monoclonal	30
COX-2 Target antigen is COX-2 in human, mouse and rat	1/1000	Rabbit polyclonal	60-80
β-actin	1/1000	Mouse monoclonal	40

Table 13: Details of primary antibodies used in Western Blotting

Following incubation with the primary antibody, membranes were washed twice in 0.1% PBS-Tween. Blots were incubated at room temperature for 1 hr with the relevant secondary antibody diluted 1/1000 in 5% BSA solution. The transferred proteins were immediately detected using ECL. Each blot was incubated for 5 minutes in ECL Western blotting reagent (Amersham, Buckinghamshire, UK) prepared according to the manufactures' instructions.

Blots were then visualised on the Trio+ Variable Mode Imager (GE Healthcare, Chalfont St Giles, UK) or by exposure on high quality X-ray chemiluminescence film (CL-XPosure), (Thermo-Scientific). A rainbow molecular weight marker (MagicMark^{TM} XP), (Invitrogen, Paisley, UK)

identified the position and molecular weight of the proteins of interest with β-actin serving as the reference gene.

2.7 Nitrate Assay

A commercial nitrite/nitrate fluorometric assay kit (Cayman Chemicals, Cambridge, UK) was used to measure the NO concentration in media from cultured primary equine cells. All reagents to measure NO expression were supplied in the kit. Media was collected from treated cells and stored at -80°C until use.

Construction of the nitrate standard curve

A nitrate standard curve was constructed from a 200 µM stock nitrate standard to measure unknown NO concentrations in samples. Serial dilutions of this stock solution were prepared with Assay buffer with NO standard concentrations ranging from 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 pmol.

Preparation of standards and samples for NO assay

A 30µl aliquot of each prepared standards was combined with 50µl of Assay buffer. A 20µl volume of each unknown sample was mixed with 60µl of Assay buffer, 10µl of Enzyme CoFactor Mixture and 10µl of Nitrate Reductase Mixture. Each 80µl standard or unknown sample was added to a 96-well plate in duplicate and incubated for 1hr at room temperature. Each sample and standard was further incubated at room temperature with 10µl of DAN reagent. An additional 20µl of NaOH was added and the plate read on a fluorometer. The excitation wavelength was set to 365nm with an emission wavelength of 430nm.

The nitrate standard curve was constructed by plotting fluorescence *vs.* picomoles of nitrate. Total NO expression in samples was calculated according to the following equation.

$$[\text{Nitrate + Nitrite}]\ (\mu M) = \left(\frac{\text{fluorescence - y-intercept}}{\text{slope}}\right)\left(\frac{1}{\text{volume of sample used } (\mu l)}\right) \text{ x dilution}$$

Chapter 3: Development of *in vitro* equine respiratory models

3.1 Introduction

In order to fully investigate RAO, recent work has focused on advancing *in vitro* techniques. To allow the investigation of the mechanism of acute inflammation and its resolution *in vitro*, methods are used to isolate respiratory cells types such as bronchial epithelial cells and airway smooth muscle cells.

The methods available to investigate equine respiratory disease *in vitro* are varied and offer their own relative advantages and disadvantages. The most suitable approach to asses RAO inflammation *in vitro* therefore requires careful assessment of the available methods.

3.2 Aims

- Develop equine *in vitro* models of bronchial epithelium and airway smooth muscle
- Characterise and assess selected models for viability and suitability for *in vitro* respiratory investigations of inflammation and inflammatory resolution

To achieve these aims, respiratory tissue from sections of equine trachea were used to establish either primary ETE or ASM cells. Primary cells were obtained from enzyme digestion of tracheal tissue or from tissue explants. Culture of both whole organ sections and specific tissue types isolated from the trachea were also examined as potential *in vitro*

respiratory models. Suitable models were characterised and assessed for viability by:

i. The trypan blue test for primary cells
ii. Evidence of ciliary movement in tissue models by use of Polysphere beads, or by visual inspection of appearance in whole organ models.
iii. Cell type and morphology in isolated primary cells were identified by ICC, IF and IHC methods.

3.3 Primary cell and explant cultures

Source of material

Fresh equine tissue was obtained from a) a local abattoir (Red Lion, Nantwich), b) the Defence Animal Centre (DAC) in Melton Mowbray, or c) clinical cases euthanased at the School of Veterinary Medicine and Science at the University of Nottingham. Tissue was acquired from horses of both sexes with ages ranging from 3 to 29 years old. Age of the horses was determined by either passport details (DAC and Vet School) or approximate dental ageing (abattoir). Horses euthanased at the DAC and the Veterinary School were determined to be free from systemic disease (based on clinical history) with no known inflammatory conditions or recent administration of medication.

To ensure that healthy tissue was used in experiments, the carcass from all sources was inspected for any signs of systemic disease, including mucopurulent nasal discharge suggestive of bacterial respiratory infection. The trachea was dissected free immediately after slaughter and examined

for signs of gross pathology such as respiratory disease. This included mucosal surface inflammation, evidence of mucopus, excessive mucus production and primary tracheal lesions (considered uncommon in the horse).

Tissue collection

Isolated equine trachea was washed extensively in sterile cell culture grade ice-cold PBS (Invitrogen, Paisley, UK). Tissue collected from the abattoir was transported on ice back to the laboratory in PBS containing 1% (v/v) of Pen/Strep (10,000U/ml//10,000µg/ml respectively) (Invitrogen, Paisley, UK) and 1% (v/v) 250 µg/ml Fungizone (Amphotericin B in water), (Invitrogen, Paisley, UK). Maximum transport time of abattoir tissue was approximately 2 hours.

Tissue preparation

All tissue preparation and dissection was performed under a laminar flow hood under aseptic conditions. The adventitia and any adherent tissue attached to the exterior of the trachea were removed. Approximately 15-20cms of trachea was opened longitudinally, by sharp dissection, for further dissection or tissue isolation. All steps requiring rinsing of isolated tissue were performed using sterile cell-culture grade PBS containing 1% (v/v) of 10,000U/ml/10,000µg/ml Pen/Strep and 1% (v/v) 250µg/ml Fungizone (Amphotericin B in water) unless otherwise described.

3.4 Isolation of primary equine tracheal epithelial cells using trypsin

Primary tracheal epithelial cells were isolated from the trachea after the method described by Shibeshi *et al* (2008) with minor modifications.

Tissue preparation was as described. The epithelial layer was removed from the mucosa using serrated edge forceps. Isolated tissue was macerated manually using scissors into 1-2mm^{3}pieces and approximately 500µg of tissue placed in a disposable sterile 50ml Falcon tube containing 20mls of sterile PBS (Invitrogen, Paisley, UK). The tissue was washed 3-4 times in fresh sterile PBS by centrifugation for 1 minute at 1200 rpm (300g), 15°C to eliminate contaminating red blood cells.

Minced washed tissue was then placed into 20mls of warmed (37°C) F-12 Nutrient Mixture (Hams), (Invitrogen, Paisley, UK) containing 0.25% porcine trypsin/2.65mM EDTA (Sigma-Aldrich, Poole, UK), 1% (v/v) of 10,000U/ml/10,000µg/ml Pen/Strep and 1% (v/v) 250µg/ml Fungizone (Amphotericin B in water) (Invitrogen, Paisley, UK). Tissue samples were incubated in a water bath tissue for between 30mins and 1 hour (37°C) and agitated regularly to enhance enzymatic digestion.

Following enzymatic digestion, supernatants were aspirated from each sample and enzyme action halted by the addition of 20% (v/v) heat-inactivated FBS, (Fisher Scientific, Loughborough, UK) to each sample. Samples were pelleted by centrifugation at 300g for 10 minutes at 15°C. Cell pellets were re-suspended in 20mls of warmed (37°C) sterile PBS and filtered through disposable sterile 40µl cell strainers. Cell suspensions were again centrifuged at 300g for 10 minutes at 15°C. The cell pellet from each sample was re-suspended in 1ml of warmed (37°C) serum-free BEGM, (Lonza, Slough, UK).

Following a cell and viability count, cells were seeded at 6-8 $x10^5$/ml in uncoated $25cm^3$ tissue culture flasks or 6-well uncoated tissue culture plates. Cell cultures were maintained in a tissue incubator in humidified 5% CO_2 at 37°C to allow for attachment and growth. Cells were inspected after 24 hours for signs of contamination. Non-adherent cells or contaminated cells were discarded. Spent media was removed from viable healthy cells and fresh media added. Primary cells isolated by trypsin digestion of epithelial tissue approached 70-80% confluency within 2-5 days of being cultured.

3.5 Isolation of primary equine tracheal epithelial cells using pronase

Tissue was collected and prepared as described above. Tracheal epithelial cells were isolated according to the method of Quintana *et al* (2011). Segments of whole trachea were dissected into 1 cm x 6cm sections. Approximately 2-3 sections of dissected trachea were placed into a 50ml Falcon containing 30mls of sterile PBS supplanted with 1% (v/v) of Pen/Strep and Fungizone (Invitrogen, Paisley, UK). Tracheal sections were washed for 45 minutes at 4°C on a roller. The PBS was discarded and this step repeated.

Tissue was digested in 0.2% pronase (*Streptomyces Griseus*, Sigma-Aldrich) in 10mls of F-12 Nutrient Mixture (Hams) containing 1% (v/v) of 10,000U/ml/10,000µg/ml Pen/Strep and 1% (v/v) 250µg/ml Fungizone (Amphotericin B in water) (Invitrogen, Paisley, UK), at 4°C overnight on a roller. Enzyme action was halted; the supernatant and cell samples were

processed and collected as before by centrifugation to obtain a cell pellet. A viability and cell count was performed.

Cells were again cultured at 6-8 x10^5/ml in serum-free BEGM in 25cm^3 collagen-coated tissue culture flasks or 6-well collagen-coated polystyrene tissue culture plates. Isolated cells were cultured at 37°C, 5% CO_2 with media changed 24 hrs after collection with fresh media every 2-3 days thereafter. Cells successfully isolated with pronase typically approached 70-80% confluency between 5 -14 days.

3.6 Primary tracheal epithelial cells from explants of tracheal epithelial tissue

Sections of equine tracheal epithelial tissue were removed from the prepared trachea using rat tooth forceps. These strips were placed apical side up onto a 144 x 21mm disposable tissue culture plate and rinsed again with sterile, culture grade PBS. Isolated epithelial tissue sections were dissected further into approximately 1cm x 0.25cm sections and allowed to adhere onto uncoated 6-well tissue culture plates. Two mL of serum-free BEGM media was added to each well using disposable RNAse/DNAse-free 1000µL tips (**Fig.10**).

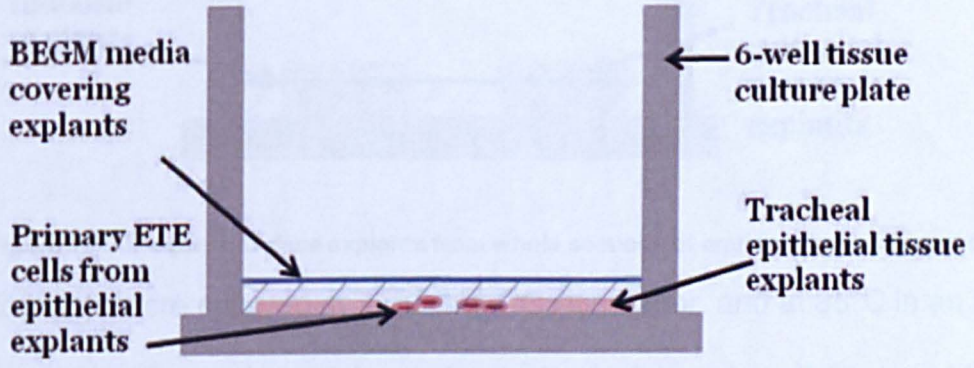

Figure 10: Primary equine epithelial cells cultured from equine mucosal explants

The tracheal mucosal explants were cultured at 37°C, 5% CO_2 in a CO_2 incubator with media changed after 24 hours. Media was changed every 48 hours thereafter. Explants were left for up to 2 weeks to allow for growth of respiratory epithelial cells.

3.7 ALI tracheal cultures

ALI cultures from explants of isolated equine epithelial mucosal tissue

Tissue preparation and collection was as previously described. Tissue explants of whole tracheal mucosa were dissected from the tracheal cartilage using sterile disposable scalpels. Isolated sections were dissected further into 2cm x 2cm pieces and washed 2-3 times with sterile PBS. Prepared explants were placed into 6-well tissue culture plates, allowed to adhere and 1ml of fresh BEGM media (Lonza, Slough, UK) was added using RNA/DNA-free 1000µL tips (**Fig.11**). This ensured that the media was in contact with the tissue but did not cover it.

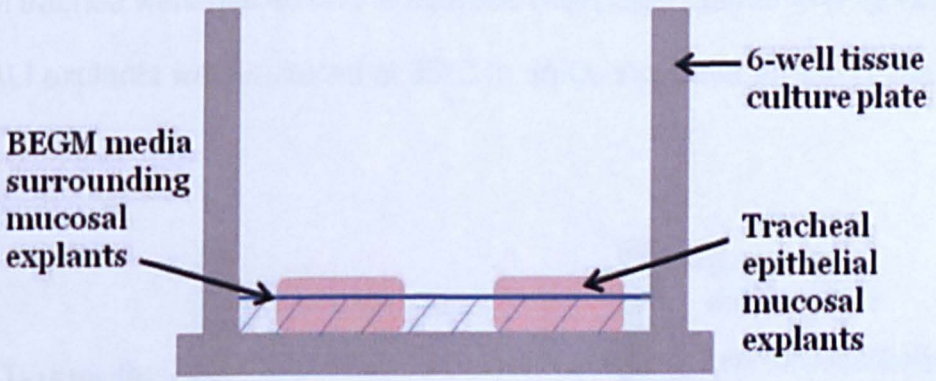

Figure 11: Air-liquid interface explants from whole sections of equine respiratory mucosa

Explants were cultured at 37°C in a CO_2 incubator, and at 35°C in an O_2 incubator. The surface of the explants was moistened each day using the surrounding media.

ALI cultures from explants of whole tracheal sections

Whole tracheal explants were prepared by dissecting sections of trachea into 2cm x 1cm pieces. The ALI cultures were prepared according to the method by Hamilton *et al* (2006).

Platforms of 1% agarose gel were constructed by dissolving agarose powder in cell-culture grade PBS (Invitrogen, Paisley, UK). The agarose solution was heated in a microwave until evenly dissolved and 1ml of prepared solution placed in each well of a 12-well tissue culture plates until set. The solidified agarose plugs were removed using a sterile disposable scalpel and placed into 6-well tissue culture plates surrounded by 2ml of L-15 medium (Fisher Scientific, Loughborough, UK).

A 1cm x 2cm strip of autoclaved filter paper soaked in L-15 medium was placed on top of each agarose platform. Strips of sterile filter paper (Whatman plc, Maidstone, UK) under the explants were allowed to touch the surrounding media to keep the explants moist. The prepared sections of trachea were placed on the agarose/filter paper platforms (**Fig.12**). The ALI explants were cultured at 35°C in an O_2 incubator for 24-72 hours.

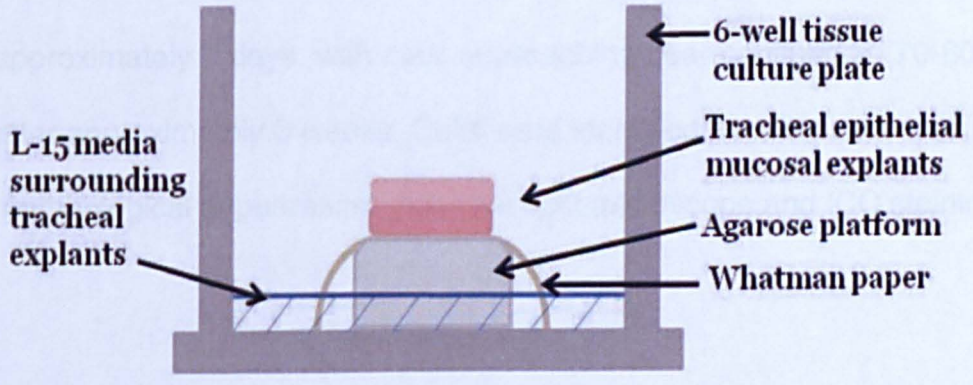

Figure 12: ALI of whole tracheal explants.

3.8 Primary airway smooth muscle cell culture from equine trachealis muscle explants

A 25cm^2 section of equine trachea was collected and prepared under the conditions as described previously. The trachea was opened longitudinally, and the mucosa covering the trachealis muscle removed using sterile forceps. The trachealis muscle was dissected from the trachea by using serrated edge forceps and any remaining adherent mucosa or connective tissue removed. The excised muscle trachealis muscle was washed on a 144 x 21mm tissue culture plate.

The trachealis muscle was furthered dissected into 1mm x 1mm explants using sterile disposable scalpels. Either 1 or 2 ASM explants were placed into each well of an uncoated Nunc 6-well tissue culture plate and allowed to adhere for up to 5 minutes. Explants were covered with 2mls D-MEM (high glucose), (Fisher Scientific, Loughborough, UK) containing 10% FBS and supplemented with 1% (v/v) of 10,000U/ml/10,000µg/ml Pen/Strep and 1% (v/v) 250µg/ml Fungizone (Amphotericin B in water) (Invitrogen, Paisley, UK).

The ASM explants were cultured at 37°C, 5% CO_2 in a CO_2 incubator. Media was changed after 5 days. Cell growth was apparent after approximately 7 days, with cells approaching near-confluency (70-80%) after approximately 3 weeks. Cells were identified as ASM cells through morphological appearance under the light microscope and ICC staining.

3.9 Cell count, assessment of cell viability and evaluation of cell type

Cell count and viability

Freshly isolated ETE cells and ASM cells were isolated and cultured as described. Cell count and viability of ETE and ASM cells count was determined with the Trypan blue exclusion test. Adherent cells from 2 wells of a six-well plate were dislodged with trypsin, centrifuged to obtain a cell pellet and re-suspended in 1ml of cell culture media, before being manually counted as described.

A 1:2 dilution was prepared with 10µl of 0.4% Trypan Blue and 10µl of original cell suspension. Viable cells were recognised by appearing colourless, cells that had taken up the Trypan blue stain were considered non-viable. An average cell count from the 1: 2 dilution of cell suspension and Trypan blue was used to calculate the cell count/viability per ml.

Viability of epithelial explants

Viability was assessed through visible cilia movement of the explants as viewed under a light microscope.

Viability of ALI tracheal explant sections

The viability of the ALI culture system was assessed through activity of the respiratory mucocillary escalator. A 10µL sample of Polybead Microsphere® beads (Polysciences, Northampton, UK) was placed onto the middle of an explant section. Movement of the beads to the edge of the tissue within 1hr after incubation at 35°C indicated that the tissue was viable. Healthy active tissue was also indicated by the change of media colour after 24 hours of incubation.

Characterization of primary ETE and ASM equine cells

Primary ETE and ASM cells were identified by a combination of morphology and ICC staining. Positive and negative controls included IHC staining of paraffin embedded tissue sections (**Table 14**). Negative controls were incubated with the matching isotype control for each primary antibody used. All ICC/IHC samples were washed three times for 5 min in 0.1% Tris Buffered Saline-Tween (TBS-Tween, 137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20, pH 7.6) after each step. All media and reagents were removed before continuing to the next stage of the protocol.

Preparation of primary cells for staining

Primary ETE and ASM cells were collected and isolated as before. Both ETE and ASM cells were cultured to 70-80% confluency either in the 6-well tissue culture plate or on sterile 13mm disposable plastic cover slips (Sarstedt, Leicester, UK).

Cells were fixed prior to staining by incubation in an ice-cold 1:1 solution of acetone-methanol for 10 minutes. Use of acetone-methanol enables fixation of antigens while retaining cellular and subcellular structure. Acetone-methanol fixation also enables permeabilisation of cells to allow full access to the target protein by the labelling antibodies. Use of formaldehyde fixation may induce cross-linkage of proteins and prevent detection of the antigen, thus requiring a subsequent antigen retrieval step.

Preparation of tissue sections for staining

Paraffin embedded tissue sections of either rat kidney and mouse colon were initially immersed for 5 minutes (x2) in fresh xylene baths. The tissue sections were a kind gift from Dr Katie Asplin (Liphook Equine Hospital, Hampshire). The tissue sections were then taken through a series of

alcohol baths (100-50%) with an incubation time of 2 min per bath. The de-waxed sections were then used immediately in the blocking steps.

Inhibition of peroxidase activity and blocking step

Samples were incubated in 3% H_2O_2 solution (30% H_2O_2 diluted in PBS) for 20 minutes at room temperature to inhibit endogenous peroxidase activity. Non-specific binding was blocked by incubating all samples in 10% BSA solution diluted in 0.1% PBS-Tween for 1 hour at room temperature.

Incubation of samples with antibody and development of IHC/ICC stain

Following aspiration of the BSA solution, samples were incubated overnight (12-14hrs) at 4°C with the relevant primary antibody or matching isotype control (**Table 14**).

Primary antibody (Ab)	Ab isotype	Species primary Ab was raised in	Protein Ab reacts with	Positive tissue control
Pan-cytokeratin 26	IgG1, monoclonal	Mouse (anti-human)	Cytokeratin 5,6 and 8 in simple squamous epithelia and pseudostratified epithelia	Rat kidney: able to express cytokeratin 8
Alpha smooth muscle actin (Clone 1A4)	IgG2a, Monoclonal	Mouse (anti-human)	Cytoplasmic α-actin isoforms present in smooth muscle cells	Mouse colon: able to express α-actin

Table 14: Primary ICC/IHC antibodies and isotypes used in primary equine cell characterization

Two to three drops of the secondary polymer Horse Radish Peroxidase (HRP) antibody (Dako, Ely, UK, supplied in kit) were then applied to each sample and the sample incubated for 30 minutes at room temperature.

This step incorporates a modified labelled avidin-biotin step method which enables a biotinylated secondary antibody to form a complex with peroxidase-conjugated streptavidin molecules **(Fig.13)**

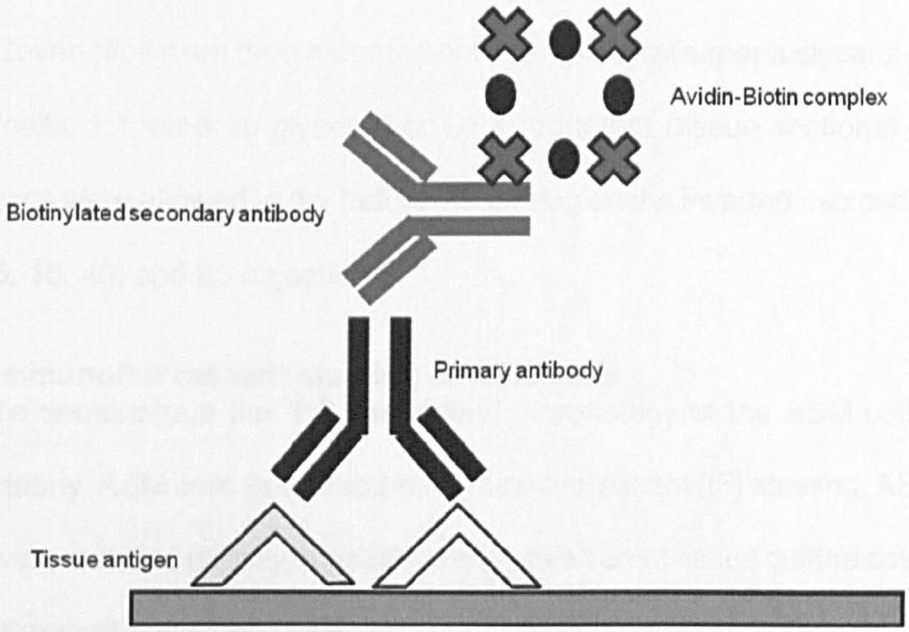

Figure 13: Representative image of complex formed between avidin and biotin during IHC/ICC staining.

(Figure adapted from www.dako.com)

Samples were washed with PBS-Tween (5 minutes x 3) after both primary and secondary antibody incubation steps. Each sample had 2-3 drops of chromogen developing solution (1 drop of chromagen to 1 ml of substrate buffer) applied (Dako, Ely, UK, supplied in kit) and left until brown staining was visible to the eye. The reaction was halted by rinsing the developed sample under tap water. Samples that did not obviously stain within 5 minutes were left for up to 30 minutes to account for slow development of stain.

Samples were then incubated in haematoxylin for 10 seconds (primary cells), or 1 minute (tissue sections) to counter-stain the nucleus, rinsed

clear in tap water, with a final wash in ammoniated water for 1-2 seconds. Tissue sections were taken through a series of alcohol baths (50-100%, 20 seconds per bath) to dehydrate and fix the developed stain.

Cover-slips were then mounted onto samples, by either a glycerol solution (cells, 1:1 water to glycerol) or DPX mountant (tissue sections). Cover-slips were allowed to dry before visualising on the inverted microscope at x 5, 10, 40, and 63 objectives.

Immunofluorescent staining of ASM cells

To demonstrate the "hill and valley" morphology of the ASM cells more clearly, ASM cells were used in immunofluorescent (IF) staining. ASM cells were cultured directly on sterile disposable 13mm tissue culture cover slips (Sarstedt, Leicester, UK).

When cells were approximately 80% confluent, the culture media was aspirated and samples washed twice for 5 minutes in warm cell-culture grade PBS. The cell samples were fixed in 4% formaldehyde for 10 minutes at room temperature, and then the PBS wash step repeated. To reduce background staining, the fixed cells were incubated for 30 minutes in a 1% BSA/PBS solution for 30 minutes at room temperature. A PBS wash step was repeated twice.

Each sample was then incubated for 20 minutes at room temperature in 1/100 Alexa-Fluor 488 solution (Invitrogen, Paisley, UK). Alexa-Fluor 488 phalloidin is a probe conjugated to green-fluorescent Alexa-Fluor 488 dye which stains filamentous actin in samples (specifically F-actin, a microfilament present in smooth muscle). Samples were then incubated for

3 minutes at room temperature in a DAPI solution (Fisher Scientific, Loughborough, UK). Samples were fixed onto glass slides and sealed using a 1:1 ratio of glycerol and double distilled water.

3.10 Results

Cell count and viability of primary cells

Cell isolation with pronase
Isolation of primary ETE cells with pronase was unsuccessful. Low cell yield, with weak adherent capabilities and slow growth indicated that this method was not sufficiently reliable for use in future work (based on 4 individual experiments).

Cell isolation with trypsin
Trypsin-dissociated primary ETE cells averaged a mean cell count of 173 x 10^4 (SEM ± 36) cells per ml from 5mg of minced tissue. Cell viability was estimated to be 78.6% (SEM ±3.0, calculations from 7 separate experiments, performed in triplicate). Isolated cells were maintained as submerged monolayers on collagen-free tissue culture plates. Near confluence of primary cell cultures was frequently apparent after 3-5 days **(Fig.14)** and cells exhibited typical "cobblestone" epithelial cell morphology.

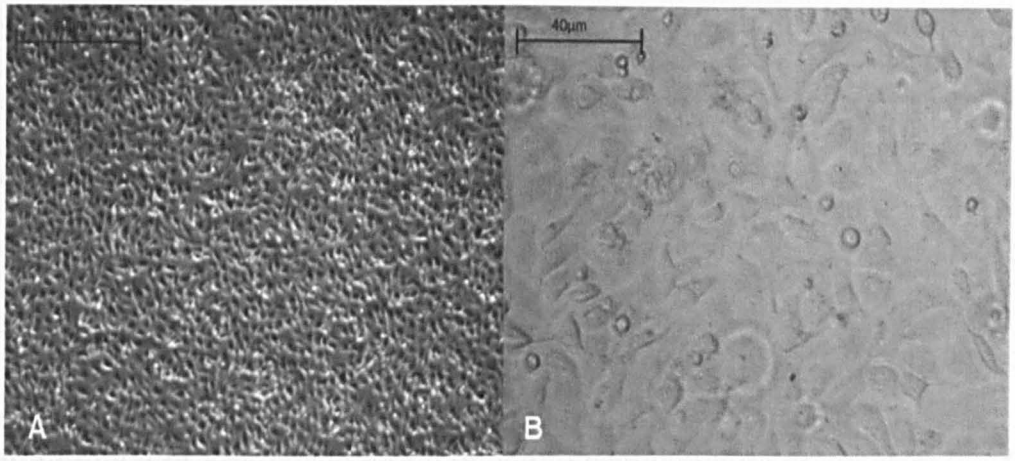

Figure 14: Bright field image of primary ETE cell cultures obtained from trypsin digestion of epithelial tissue.

Representative images show ETE cells maintained on collagen-free 6-well tissue culture plates after 3 days culture (A, x10 magnification) and after 10 days culture (B, x40 magnification). Images are representative of 2 independent experiments and selected from triplicate samples.

Viability of primary ETE cells from epithelial tissue explants

Sections of tracheal epithelial tissue explants successfully adhered to the tissue culture plate (**Fig.15**). Ciliary movement signifying tissue viability was observed under the light microscope within 1hr of establishing cultures and continued for up to 7 days thereafter.

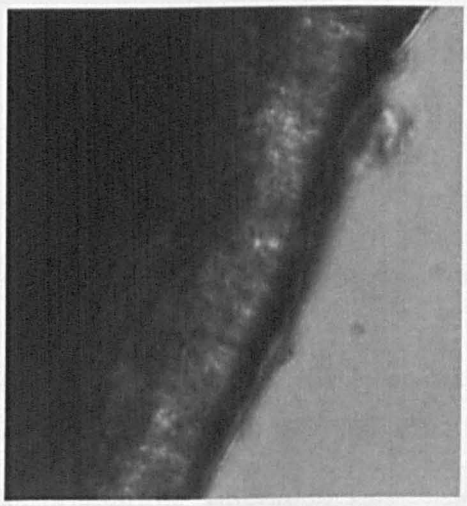

Figure 15: Explant of equine tracheal epithelial tissue after 6 days in culture.

Explants were cultured in BEGM media on collagen free tissue culture plates for up to 7 days to allow for outgrowth of primary ETE cells.

Cultures with no apparent ciliary movement after 24hrs were discarded (based on three separate experiments). Primary cells surrounding the explants were observed 24hrs after the initial culture set up. No further growth of these cells was evident after 7 days and this model was not used for further experiments.

Viability of ALI sections of equine respiratory mucosa

Adherence of ALI explants of mucosa to tissue culture plates was poor. Explants frequently detached during initial isolation and culture maintenance. Explant tissue appeared dry, and red in colour (indicating inflammation) despite daily bathing of sections in culture media. As such this model was deemed unsuitable for further study.

Viability of ALI cultures from explants of whole tracheal sections

Ciliary action indicating explant viability was determined through movement of Polybead Microsphere® beads (Polysciences) placed on tracheal sections. No bead movement was observed during initial set up or when repeated at 24hrs. Some metabolic activity was apparent from some loss of colour from the media after 24hrs indicating minor activity. Despite this, cultured explants were not considered to be sufficiently active or viable. This method was also deemed to be unsuitable for future work (based on four separate experiments).

Characterization of primary ETE cells

Primary ETE cells isolated with trypsin demonstrated the highly typical 'cobblestone' epithelial cells morphology described in the literature. Primary ETE cells were further characterised with ICC and IHC methods **(Fig.16)**

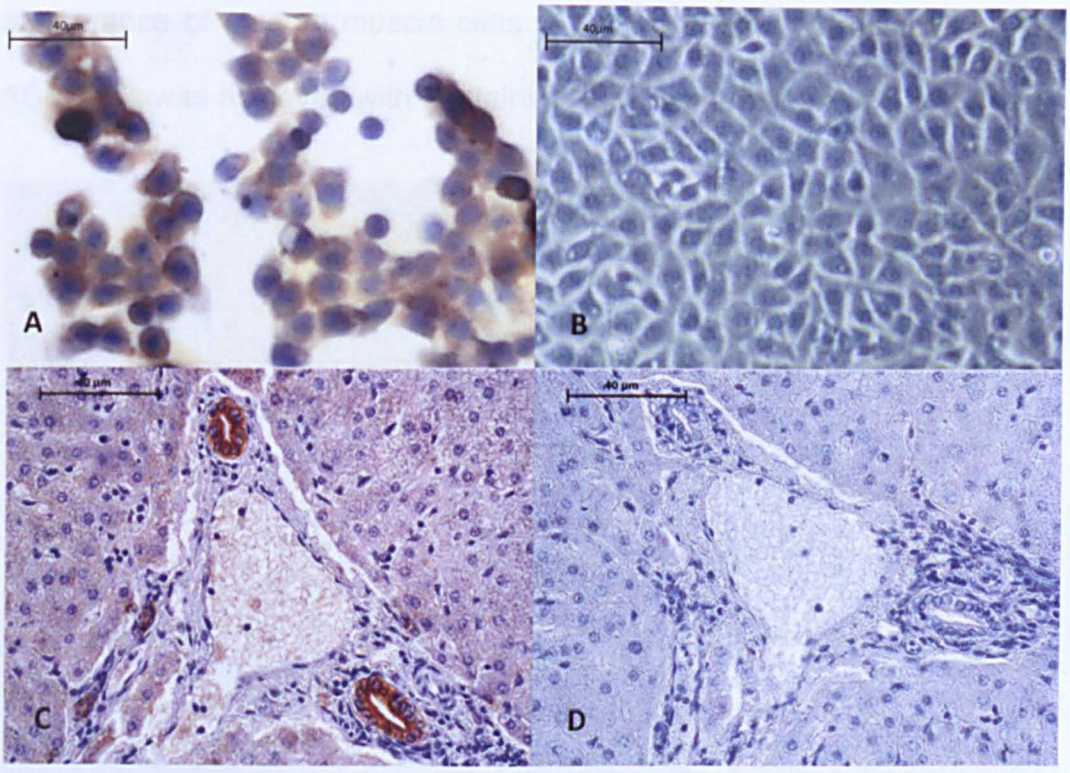

Figure 16: Bright field images of ICC and IHC stain of primary ETE cells and rat kidney tissue sections.

Representative images show pan-cytokeratin (PCK-26) stain of primary ETE cells (A) and IgG1 isotype control (B). Paraffin embedded sections of rat kidney acted as positive control for pan-cytokeratin (C) with isotype control also shown (D). Primary ETE cells were isolated from 2hr trypsin digestion of epithelial tissue. Images are representative of 3 independent experiments and selected from triplicate samples (A & B: x40 magnification).

Cultured cells and the paraffin-embedded tissue sections of rat kidney stained positive for pan-cytokeratin antibody PCK-26. Immunostaining of cells and tissue sections with the corresponding IgG isotype (negative control) did not show evidence of staining. It was concluded that these were viable cells of epithelial origin and highly suitable for the proposed work investigating equine respiratory inflammation and resolution.

Characterization of primary ASM cells

Cells isolated from explants of trachealis muscle became near-confluent after approximately 3 weeks. Initial detection of ASM cell morphology used a basic H and E stain which revealed the characteristic 'hill and valley'

appearance of smooth muscle cells in culture. Further investigation of ASM cells was achieved with IF staining with Alexa-Fluor 488 (**Fig.17**).

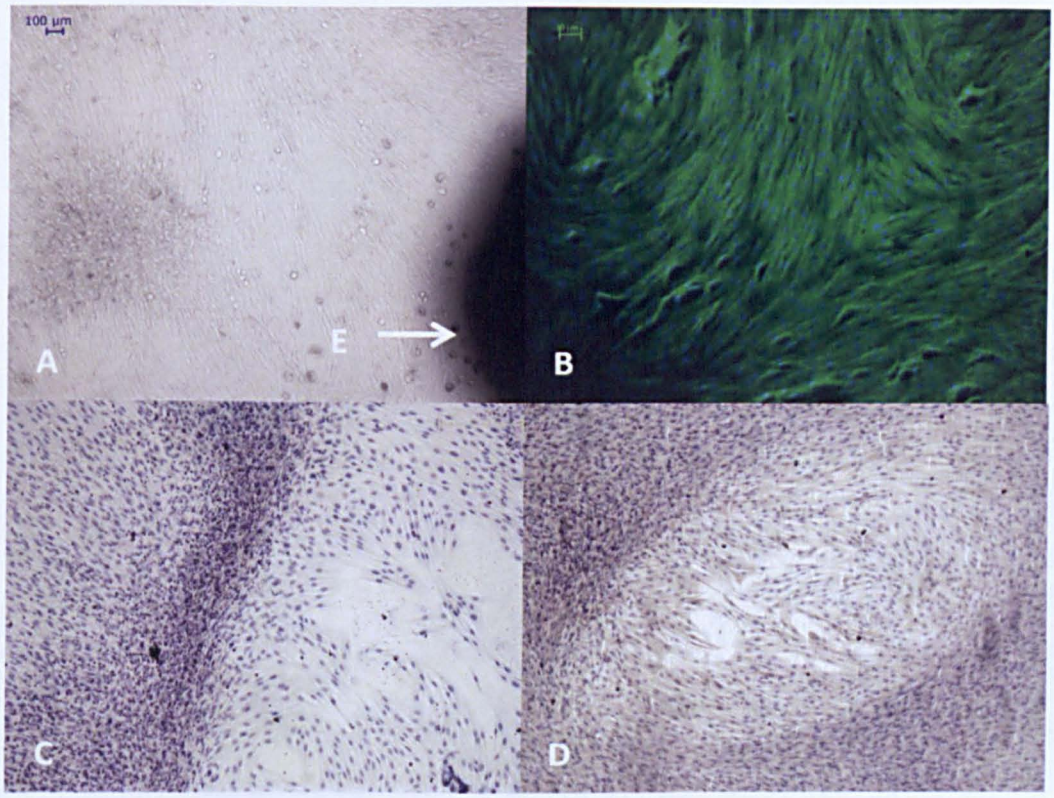

Figure 17: Bright field images of IF and H and E stain of primary equine ASM cells

Representative images show primary equine ASM cells obtained from trachealis muscle explants approximately 3 weeks after initial set up. Images with (A) shows ASM cell outgrowth from explant (E). ASM morphology is shown in greater detail in image B. The 'hill and valley' morphology considered typical of smooth muscle cells is demonstrated in images C and D by a standard H and E stain. For the H and E stain, ASM cells were maintained in untreated 6-well tissue culture plates for 16 days. Images are from 3 individual experiments and selected from triplicate samples.

Alexa-Fluor 488 labels F-actin microfilaments present in smooth muscle and enabled a more clearly defined image of AS cell morphology. To further identify isolated cells as smooth muscle cells, ICC and IHC methods were used. Cultured ASM cells and the paraffin-embedded tissue sections of mouse colon (included as a positive control) stained positive for smooth muscle actin, a protein marker for smooth muscle tissue.

Treatment of cells and tissue sections with the corresponding isotype (negative control) did not show evidence of staining (**Fig.18**).

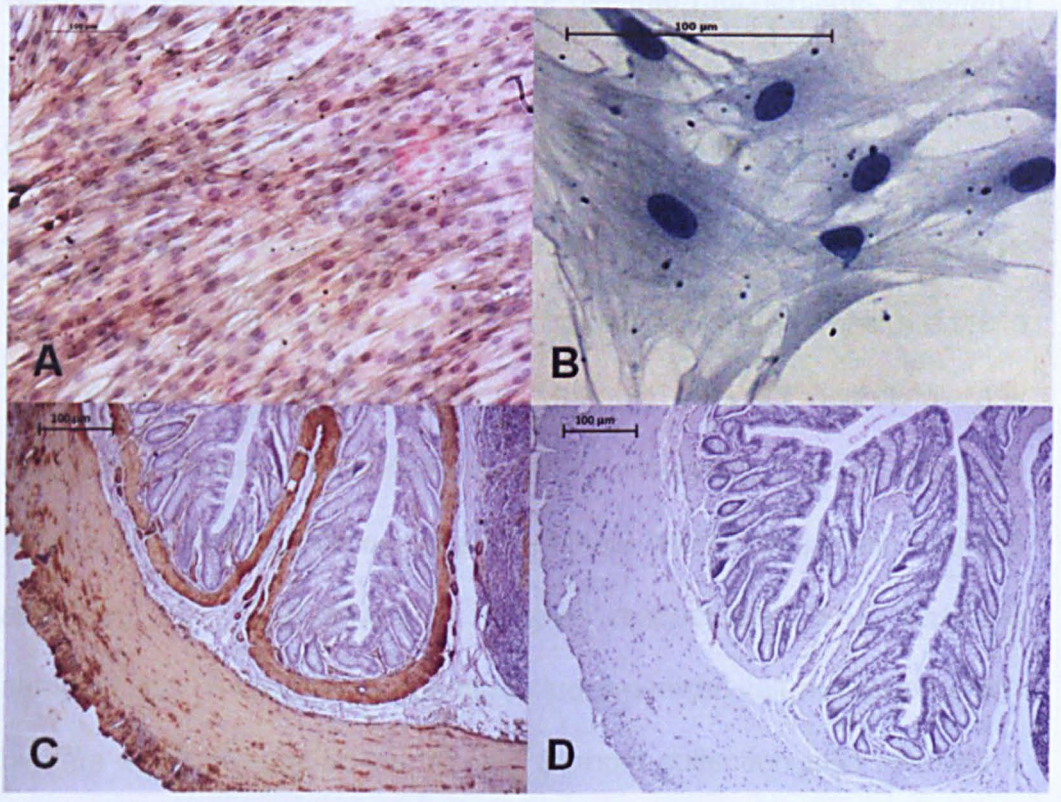

Figure 18: Bright field images of ICC and IHC stain of primary ASM cells and mouse colon tissue sections.

Representative Images show α-smooth muscle actin (clone 1A4) stain of primary ASM cells (A) and IgG2a isotype control (B). Paraffin embedded sections of mouse colon acted as positive control for α-smooth muscle actin (C) with isotype control also shown (D). Images show primary equine ASM cells obtained from trachealis muscle explants cultured on untreated 6-well tissue culture plates approximately 3 weeks after initial set up. Tissue samples of Images are from 3 independent experiments and selected from triplicate samples.

It was concluded that the cultured cells obtained from trachealis muscle explants were of smooth muscle origin and suitable for use in future work investigating ASM cell inflammation and inflammatory resolution.

Discussion

Respiratory models to investigate asthma in humans frequently use *in vivo* techniques. In contrast, equines are considered poor *in vivo* subjects, in

terms of cost and ease of maintenance. *Ex vivo* or *in vitro* methods eliminate the cost and difficulties associated with the use of experimental horses and enable the examination of disease mechanisms. Research using *ex vivo* techniques enables investigative opportunities that would be otherwise impossible or unethical to perform in the living animal.

Investigations of equine airway inflammation use a variety of *ex vivo* techniques. Using samples of BAL or tracheal aspirate (TA) is a reliable and repeatable method and offers valuable data for detecting early respiratory inflammation (Hoffman 2008). However, a study using 28 horses indicated that bronchial and tracheal brushings contain bacteria and fungi (10.7 and 53.6% of samples respectively). BAL, brushing samples and TA samples frequently includes a mixed cell population which hinders study of specific cell types. Sample collection also involves a degree of invasiveness when performed (Leguillete & Lavoie 2006). Lung tissue samples are considered representative of the whole lung and are also used extensively in diagnosing equine respiratory disorders (Lugo *et al* 2006). Whilst useful, *ex vivo* methods were not possible for investigation into equine respiratory inflammation due to the lack of available horses for study.

During the development of an *in vitro* equine respiratory model of the respiratory tract, several approaches were used

i. primary epithelial cells isolated from digested respiratory tissue
ii. primary airway smooth muscle cells from explants of trachealis muscle

iii. primary epithelial cells from tracheal explants under submerged conditions

iv. mucosal explants kept at the air-liquid interface

v. whole organ explants cultured at the air-liquid interface

An *in vitro* equine epithelial model was successfully established by trypsin digestion of airway epithelial tissue to obtain primary equine ETE cells, and explants of trachealis muscle to culture primary ASM cells. Primary ETE and ASM cells were identified by IHC and ICC staining with pan-cytokeratin antibody PCK-26, and α-smooth muscle actin (clone 1A4) respectively. These antibodies identifies cytokeratin proteins 5, 6 and 8 found in simple squamous epithelia and pseudostratified epithelia (PCK-26), and cytoplasmic α-actin isoforms present in smooth muscle cells (α-smooth muscle actin).

Primary ETE cells were obtained by use of the method by Shibeshi *et al* (2008) with modifications, which routinely isolated viable and rapidly proliferating tracheal epithelial cells. Viability of isolated ETE cells was estimated to be 78.6% (SEM ±3.0) and cultures were maintained in serum-free BEGM media until near-confluent at 3-5 days. The original methodology describes an average of 5×10^4 cytokeratin-positive epithelial cells obtained per mg of tissue. In contrast, average numbers of cells isolated with the original method during ETE cell model development were 3.4×10^4 per mg of tissue. Viability (as determined by the trypan blue exclusion method) was also reduced in comparison to the original method, with an average of 78.6% SEM ±3.0 compared to 9%. This may have been due to less replicates performed compared to the original paper. However,

unlike the original study, it was not found necessary to remove contaminating fibroblasts by a phase of pre-plating prior to the culturing period.

Other *in vitro* models attempted during the course of this study proved unsuitable. Studies using pronase digestion of respiratory tissue have proved successful in different species (You *et al* 2002, Rowe *et al* 2004, Fischer *et al* 2007). Primary equine epithelial cells dissociated from pronase digestion have been reported before (Sime *et al* 1997, Ainsworth *et al* 2009, and Lin *et al* 2001).

In contrast to these studies, the unsatisfactory cell yield and viability obtained from pronase digestion of respiratory epithelial tissue were unexpected. Increasing the pronase concentration and incubation times (up to 18hrs, data not shown) did not improve cell yield nor did the addition of supplemental digestion media such as collagenase. Inspection of tissue at approximately 18hrs under the light microscope showed very few dissociated cells, and it was decided not to continue. Attempting alternative methods such as shorter digestion times (1-2hrs) of epithelial tissue using pronase but with an increased temperature (37°C) were also unsuccessful. One approach not tried was the inclusion of 0.1% deoxyribonuclease to 1.4% pronase as successfully described by Quintana *et al* (2010).

Attempts to enhance cell viability by increasing the adherent capabilities of pronase-dissociated cells were not improved by pre-coating tissue culture flasks with collagen, or by using commercially pre-coated tissue culture

flasks. Cell growth may have been improved by leaving cells for longer periods in culture, as reported by Sime *et al* (1997). However, comparable more recent works (Lin *et al* 2001, Ainsworth *et al* 2009, Quintana *et al* 2011) report robust cell growth after approximately 5 days and this was taken as an indicator of superior cell viability.

One potential factor in tissue viability and subsequent cell yield may have been the length of time to transport and process collected tissue samples. Primary ETE cells have been obtained following digestion of bronchial tissue in 1% Protease XIV with 0.01% DNase (Schwab *et al* 2010). Workers on this study were able to process collected tissue samples within 1hr of death which was not an option for our particular investigation. Our total time for tissue collection and preparation averaged >2hrs and this could have affected primary cell viability and yield.

Attempts to improve poor cell growth after pronase isolation were addressed by considering culture conditions. Attempts to enhance cell proliferation and growth by alterations to the culture media were considered but not thought to be required. Cell cultures were maintained in BEGM serum-free media which has been formulated for respiratory cell culture. This media is recommended for proliferation and maintenance of the continuous bronchial cell line BEAS-2B, and in other works requiring serum-free media for bronchial cells. Enhancing cell media with serum supplementation (1%) is reported to improve initial outgrowth of primary cells from lung tissue explants at 10-14 days in culture (Kitamura *et al* 1990). In contrast, serum-enriched media (10%) increased COX-2 protein

and mRNA in the alveolar cell line NCI-H292 (Sung *et al* 2011), which would have affected investigations into respiratory inflammation.

One alternative not attempted in our work was the use of a serum substitute such as Ultroser G (Smit *et al* 1995). This has been described in a similar equine study which isolated ETE cells with trypsin digestion (Abraham *et al* 2011). Supplementation with Ultroser G was required for cellular differentiation when primary cells were cultured at the ALI (air liquid interface). Both serum and Ultroser G have been shown to alter the fatty acid composition of the plasma membrane in HeLa cells in suspension culture (Blixt *et al* 1990). They were therefore considered unsuitable to use in LXA4 studies as LXA4 is derived from substrates contained within the cell membrane, and receptor ALX is expressed on the plasma membrane (Chiang 2002, Maderna *et al* 2010).

Efforts to obtain primary bronchial epithelial cells from tissue explants were also unsuccessful. The use of respiratory tissue explants to obtain primary cells has been routinely used in previous cell culture studies (Inayama *et al* 1995, Forrest *et al* 2005, Yaghi *et al* 2010).The frequent detachment of the explant from the tissue culture plate was a hindrance that this study was not able to eliminate. One alternative would have been the use of collagen-coated flasks (Margarit *et al* 2005), an option not explored by this investigation.

Adherent epithelial explants in our study showed an initial outgrowth of primary cells from tissue sections. Explants were monitored daily for continued evidence of cilia movement which was taken as an indicator of

viability (Jackson *et al* 1996). One concern was the integrity of explants maintained in culture over extended periods under submerged conditions. Explants were removed at 7 days or when cilia movement stopped indicating that the explant appeared no longer viable. This was to allow for cell growth without potential contamination from tissue disintegration. Primary cells obtained from explants were further maintained for approximately 14-21 days (data not shown). Despite this, cultured cells did not continue to proliferate or change morphology suggestive of epithelial cells as viewed under the light microscope (Baeza-Squiban *et al* 1991).

Improving the assessment of viability of mucosal explants and tracheal sections kept at the air-liquid-interface may have improved the quality of experimental work. The use of Polysphere beads is a reliable and cost-effective method of establishing activity of the muco-ciliary escalator in respiratory tissue (Niesalla *et al* 2009). However, it does not calculate the percentage of live/dead cell ratio. One alternative to assess cell health would have been the assessment of cell apoptosis, with TdT-mediated dUTP-X nick end labelling (TUNEL). This qualitative method determines single and double-stranded DNA breaks by the use of fluorescence microscopy (Glorieux *et al* 2011). Such methods could have indicated the suitability of collected tissue for further work. This approach would have also been beneficial for attempts at ETE organ culture

ALI methods for respiratory organ culture have been advantageous in virology studies (Lin *et al* 2001) but did not translate well for this study. Efforts to keep the surface of explants sufficiently moistened by either use of an orbital shaker or by daily bathing of tissue with culture media were

unsuccessful. Organ cultures from sections of whole trachea proved largely uncomplicated to establish during set up. Despite this, external tracheal adventitia could not be removed entirely and was considered to be a potential source of media contamination. This was particularly evident during alternative attempted methods (data not shown) of whole organ culture where tracheal sections were cultured in a rotating platform to mimic the *in vivo* state.

Further disadvantages with this method included failure to isolate high quality RNA. Aseptic removal of either the epithelial or mucosal layer proved difficult due to the reduced dimensions of organ cultures. An alternative method of scraping the epithelial surface with a disposable scalpel collected low concentrations of poor quality RNA. The interaction of the epithelial layer with tracheal smooth muscle is of significant interest for investigations of respiratory inflammation. Isolating and attributing cell reactions to either tissue type were not possible with the use of whole organ culture, and rendered it unsuitable.

The use of trachealis tissue explants to obtain primary equine ASM cells was routinely successful in generating high numbers of viable cells. Cells were observed from approximately 7 days after initial seeding of the explant, with steady growth of primary cells until confluence at 21-28 days thereafter. Unlike the challenges posed by lack of adherence seen in epithelial mucosal explants and ALI models, the smooth muscle explants remained firmly attached to the tissue culture flasks following set-up.

Primary human ASM cells have been isolated by enzymatic digestion of minced tissue (Mckay *et al* 1998). However, obtaining primary cells obtained from equine muscle explants proved to be a simple and robust technique and so other methods were not considered. Additionally, the study aimed to avoid potential baseline inflammation in cultured ASM cells as observed in primary ETE cells encountered following trypsin digestion of tissue.

A 'hill and valley' morphology in cultured ASM cells considered characteristic of smooth muscle cells (Hirst 2004) was clearly demonstrated with H and E and IF methodology. The use of alpha smooth muscle actin as a marker of ASM cells has been routinely used in studies culturing ASM cells (Hirst *et al* 2004, Stamatiou *et al* 2012). IHC of cultured primary cells revealed positive staining for alpha smooth muscle actin and on positive control tissue (mouse colon). No apparent staining was observed in matching isotype negative control cell or tissue section samples.

However, identification of primary ASM cells *in vitro* has been of increased scrutiny in more recent studies (Wenzel *et al* 2006). The reliability of protein actin as an identifying marker for smooth muscle is under review. It is noted that fibroblasts and myofibroblasts in culture also have the capacity to stain positive for this protein, thus rendering them indistinguishable from ASM cells (Wenzel *et al* 2006). Myofibroblasts can develop from fibroblasts and are typically associated with the promotion of wound healing (Shimoda *et al* 2010). Thus use of explants may constitute a form of wound healing e.g. the reaction of excised dissected tissue. Use

of serum and passaging of cells is thought to change fibroblasts to myofibroblasts (Wenzel & Balzar 2006).

There were a few but important differences in the ASM cultures that identified them as ASM cells. The 'hill and valley appearance' is not a feature of fibroblasts (Hirst 2004) and was readily observed in our H and E stains of ASM cultures. The proliferation rate of ASM cells in comparison to fibroblast cells was examined. Fibroblasts *in vitro* demonstrate considerably rapid growth (Hirst 2004). Although our cultures showed cells surrounding the explants within approximately 7 days, these cells did not differentiate and approach full growth until at least 21 days. Finally, the capacity for myofibroblasts to stain positive for smooth muscle actin is thought to be transient. IHC methods have shown that staining intensity of alpha-smooth muscle actin in myofibroblasts diminishes after 15 days (Darby *et al* 1990). Cultured near-confluent equine ASM cells demonstrated the same depth of ICC stain as that observed in IHC-treated mouse colon controls. As such, the data suggests strongly that primary cells were smooth muscle in origin.

Conclusion

I*n vitro* respiratory models were developed from the culture of trypsin-dissociated ETE cells, and ASM cells cultured from trachealis muscle explants. Primary cells characterised by IHC/ICC methods were considered to be of epithelial and smooth muscle origin and therefore suitable for investigation into respiratory inflammation.

Chapter 4: Responses of an *in vitro* tracheal epithelial model to LPS and LXA4

4.1 Introduction

An exaggerated immune response from the respiratory epithelium is a major cause of excessive acute airway inflammation in horses and people (Zhang & McDowell 1992). Failure of inflammatory resolution initiates the pathogenesis of chronic airway disease such as asthma and RAO (Berndt *et al* 2007, Buc *et al* 2009).

Previous studies have established *in vitro* inflammation in primary equine bronchial epithelial cells. Expression of molecules associated with inflammation such as IL-8, macrophage inflammatory protein and IL-1β gene expression have been detected in bronchial epithelial cells after 6 and 24hrs incubation of LPS (Ainsworth *et al* 2009, Ainsworth & Reyner 2012).

Inflammatory resolution induced by LXA4 has been investigated in human bronchial epithelial models. Human bronchial epithelial cells *in vitro* have increased LXA4 expression in response to acute acid injury (Bonnans *et al* 2006). Exogenous LXA4 has also been shown to inhibit the expression of TNF-α-induced IL-8 in human bronchial epithelial cells (Bonnans *et al* 2007). However, the effect of lipoxins in equine respiratory disease has not been fully investigated and the mechanisms remain unclear.

4.2 Aims and objectives

- Characterise the response of *in vitro* primary ETE cultures to incubation with LPS, a known mediator of inflammation
- Compare the response of LPS-treated *in vitro* primary ETE cultures with ETE cells incubated with LXA4, a molecule thought to promote inflammatory resolution

The *in vitro* epithelial model of ETE cells established from Chapter 3 was used to determine inflammation and inflammatory resolution. The effect of different concentrations of LPS at varying time points on ETE cells was assessed by expression of COX-2 mRNA. From these investigations, a time point and LPS concentration signifying inflammation was selected. The selected time point and LPS concentration was then compared to the response of ETE cells to stimulation with exogenous LXA4 to determine inflammatory resolution.

4.3 Methods

Primary ETE cells were obtained from trypsin dissociation of tracheal epithelial tissue and maintained in culture until 70-75% confluent before use in experiments. Primary ETE cells were not passaged prior to experimental investigations. Cultured cells were treated with LPS concentrations of 0.1, 10 and 100µg/ml to establish a suitable level of LPS to induce acute inflammation.

Inhibition of inflammation indicated by COX-2 mRNA levels was examined by incubation with carprofen, an anti-inflammatory drug. Expression of

COX-2 mRNA used to signify inflammation was determined by end point and q-PCR at the chosen time points of 0, 4 and 24hrs.

The response of ETE cells to LPS stimulus was compared with the effect of LXA4 treatment. ETE cells were incubated with LPS, LXA4 or pre-treated with LXA4 for 15mins before incubation with LPS. Control samples for ETE cells in all stated experiments did not receive LPS, carprofen or LXA4 treatment.

Inflammation and inflammatory resolution in ETE cells were further assessed by:

i. Quantification of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX mRNA by q-PCR

ii. Western blot detection of ALX and COX-2 protein

iii. Fluorometric analysis of NO levels

Full details of the methods used to detect inflammation and inflammatory resolution in ETE cells are described in Chapter 2

4.4 Results

End-point PCR detection of COX-2 mRNA in primary ETE cells after 24hr incubation with 10μg/ml LPS concentration

Basal and acute inflammatory states were examined in ETE cells obtained from 2hr trypsin digestion of epithelial tissue. End-point PCR was used to detect expression of COX-2 mRNA compared to reference gene GAPDH after incubation with 10μg/ml LPS for 24hrs. The chosen time point and concentration was based on similar human and horse studies of the effect

of LPS on epithelial cells (Yang *et al* 2007, Ainsworth *et al* 2009). Results were visualized with agarose gel electrophoresis which revealed PCR bands representing COX-2 mRNA to be expressed in both control and LPS treated samples (**Fig.19**)

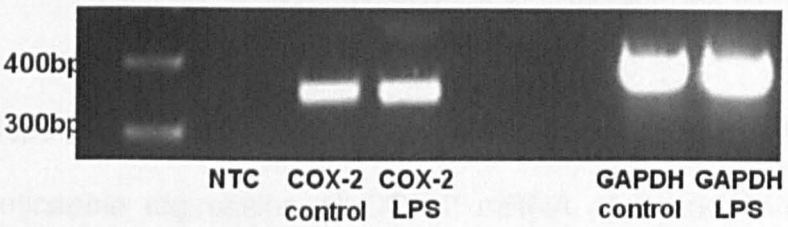

Figure 19: End-point PCR image of primary ETE cells following 24hr incubation with 10µg/ml LPS concentration.

Representative image of agarose gel shows COX-2 and GAPDH mRNA expression in primary ETE cells after 24 hrs treatment with LPS. NTC represents no template control where cDNA was omitted from PCR samples. Primary ETE cells were obtained from 2hr digestion of epithelial tissue with trypsin.

Expression of COX-2 mRNA was taken to indicate inflammation from an unknown source in both control and LPS-treated ETE cell samples. No bands were evident in the NTC control which was performed to indicate any potential contamination of PCR reaction. Contamination of PCR product would have explained the observed positive expression in the control groups. Although no such contamination of PCR products was indicated, all areas of the experimental protocols were examined for potential areas of contamination and fresh reagents and consumables were used.

The experiment was then repeated with a fresh set of primary ETE cells obtained from a different tissue donor to the previous experiment, but using the same methods and LPS concentration. In addition, a time zero sampling was included to establish if primary ETE cells were expressing

COX-2 mRNA prior to treatment. Evidence of COX-2 mRNA before LPS incubation would indicate prior inflammation in primary ETE cells which would have affected results for the control group samples.

Repeat of end-point PCR detection of COX-2 mRNA expression in primary ETE cells after 24hr incubation with 10µg/ml LPS concentration

Repeat of the experiment demonstrated that control cells still showed noticeable expression of COX-2 mRNA at 0 and 24hrs despite the absence of LPS stimulation (**Fig.20**).

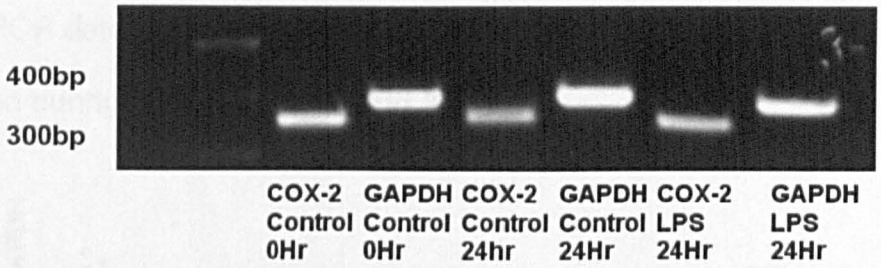

Figure 20: End-point PCR image of repeat of 24hr incubation of primary ETE cells with 10µg/ml LPS concentration.

Representative image of agarose gel shows COX-2 and GAPDH mRNA expression in primary ETE cells after 24hrs treatment with 10µg/ml LPS. Time zero was included to determine the inflammatory state of cells prior to treatment with or without LPS. Primary ETE cells were obtained from 2hr trypsin digestion of bronchial epithelial tissue from separate subject to previous experiments.

It was concluded that expression of COX-2 mRNA in untreated and treated samples at all stated time points was indicative of inflammation in ETE cells before use in experiments.

However, accurate quantification of mRNA product by end-point PCR analysis is limited. Use of agarose gel or ethidium bromide limits the level of quantative detection (Ginzinger 2002). A more sensitive method of detection was required as no allowance is made for fold-change values,

which may be as different as between 10 or 50 copies. It was possible that although COX-2 mRNA was present, levels in untreated samples were reduced compared with LPS-treated samples. It was decided to use real time PCR to ensure COX-2 mRNA in ETE cells was more accurately quantified.

Real time quantification of COX-2 mRNA in primary ETE cells after 0, 4 and 24hr incubation with 10µg/ml LPS concentration

Primary ETE cells were again dissociated from 2hr digestion of equine epithelial tissue). Time points of 0, 4 and 24hrs were included in real time PCR detection of COX-2 mRNA to indicate inflammation prior to or early on during LPS incubation **(Fig.21)**.

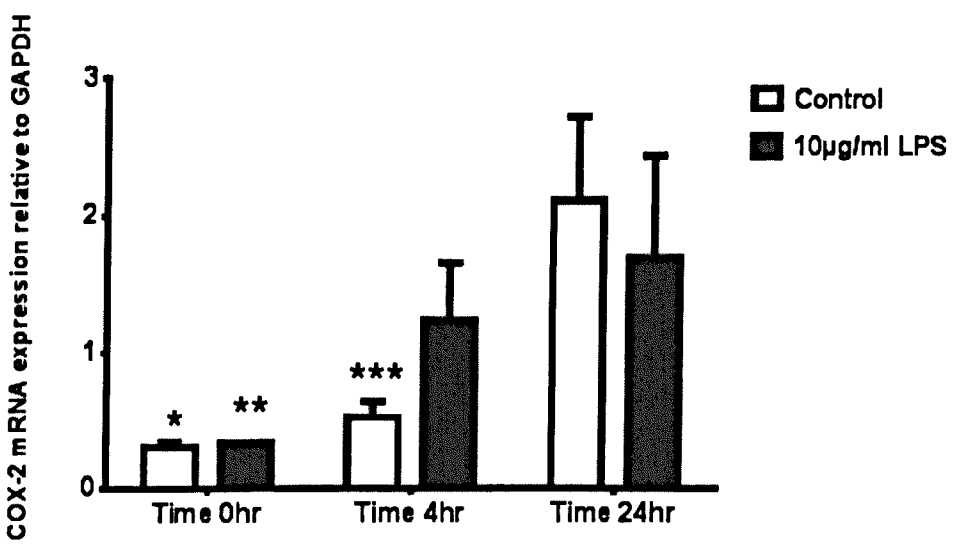

Figure 21: q-PCR graph of COX-2 mRNA in primary ETE cells after incubation with 10µg/ml LPS concentration.

COX-2 mRNA expression (relative to reference gene GAPDH) was quantified at 0, 4 and 24hrs. Primary ETE were isolated after 2hr trypsin digestion of tracheal epithelial tissue from separate subject to previous experiments. Error bars represent SEM obtained from 3 replicates from a single experiment. A two-way ANOVA was performed to indicate any statistical significance. Expression of COX-2 mRNA was significantly reduced (p value < 0.001) in control/LPS samples (*/**) at time 0hr compared with all other control/LPS samples. COX-2 mRNA was significantly reduced in control samples at 4hrs (***) compared with control samples after 24hrs (p value < 0.001)

Control COX-2 mRNA expression showed no observable difference compared with LPS-treated samples at 0hr. A two-way ANOVA indicated that control (*) and LPS-treated samples (**) at 0hr were significantly reduced compared with control and LPS treatment groups at 4hrs and 24hrs (p value <0.0001). Control samples at 4hrs (***) were significantly reduced compared with control values at 24hrs (p value <0.0001). Treatment of ETE cells with LPS did not have a significant difference on COX-2 mRNA expression.

Results suggest that LPS stimulation was not wholly responsible for any apparent increase in COX-2 mRNA expression. It was concluded that there was a steady increase of COX-2 mRNA over 24hrs in both control and LPS treated samples. The observed increases in COX-2 mRNA were attributed to inflammation caused by cell isolation or *in vitro* culture methods, rather than LPS stimulation.

To confirm the presence of underlying inflammation in isolated ETE cells, it was decided to investigate the effect of incubation of ETE cells with anti-inflammatory carprofen, a COX-2 inhibitor (Berreta *et al* 2005), with the aim of reducing basal COX-2 in ETE cells.

Effect of anti-inflammatory carprofen incubation on COX-2 mRNA expression in primary ETE cells

Fresh respiratory epithelial tissue was collected from a different horse to previous experiments and digested with trypsin for 2hrs to isolate primary ETE cells. Cell samples were incubated with either 4µg/ml carprofen, 10µg/ml LPS, or both carprofen and LPS, with samples collected at 0, 4

and 24hrs (**Fig.22**). At this point, the house-keeping gene was changed from GAPDH to 18S because it proved less variable.

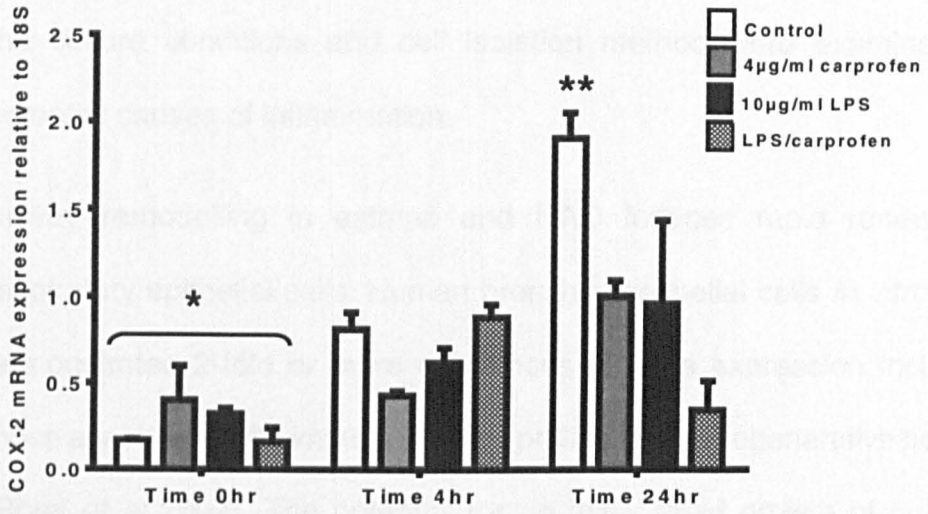

Figure 22: q-PCR graph of COX-2 mRNA expression in primary ETE cells after incubation with 4µg/ml carprofen and 10µg/ml LPS concentration

COX-2 mRNA expression (relative to reference gene 18S) was quantified at 0, 4 and 24hrs. Control samples did not receive LPS or carprofen treatment. Primary ETE cells isolated after 2hr trypsin digestion of tracheal epithelial tissue from separate subject to previous experiments. Error bars represent SEM obtained from 3 replicates from a single experiment. A two-way ANOVA was performed to indicate any statistical significance. Expression of COX-2 mRNA was significantly reduced in all treatment groups at 0hr (*) compared with all other time points (p value < 0.0001) and increased in control samples (**) compared with other treatment groups after 24hrs (** p value 0.0010)

COX-2 mRNA in all treatment groups at 0hr (*) was significantly reduced (p value < 0.0001) compared with samples at all other time points. LPS and carprofen incubation at 4 and 24hrs did not significantly increase or reduce COX-2 mRNA expression. At 24hrs, mRNA levels in control samples (**) were significantly increased (p value 0.0010) compared with other treatments at 24hrs.

It was concluded that carprofen treatment of ETE cells appeared to inhibit COX-2 mRNA after 4 and 24hrs incubation. Additionally, the observed increase in COX-2 mRNA in control samples after 24hrs indicated that pre-existing inflammation in ETE cells was present. Based on these results, the culture conditions and cell isolation method were examined for potential causes of inflammation.

Airway remodelling in asthma and RAO induces rapid renewal of respiratory epithelial cells. Human bronchial epithelial cells *in vitro* have demonstrated 2-fold or more differences in gene expression including those associated with immunity during proliferation or regenerative periods (Ross *et al* 2007). The potential for an initial rapid growth of cultured primary ETE cells isolated by trypsin treatment to develop inflammation as measured by COX-2 expression was explored.

Comparison of culture time period on inflammatory capabilities of primary ETE cells

Reports in human studies of epithelial repair indicate a return to a 'resting epithelial profile' at approximately 21-28 days after injury (Heguy *et al* 2006). Accordingly, expression of COX-2 mRNA expression was examined in primary ETE cells after either 5 days or 4 weeks in culture.

Quantification of COX-2 mRNA was obtained relative to reference gene β-actin. This change was required as cDNA synthesis at that time used oligo dT primers, which make 18S unsuitable for a reference gene due to the lack of a poly A tail. Subsequent work then used random hexamers due to low RNA concentrations extracted from cell samples. It was decided to

continue with β-actin as a reference gene for continuity in results and reliability in performance that equalled that of 18S. ETE cells were obtained from 2hr trypsin digestion of epithelial tissue collected from a different horse to previous experiments, and COX-2 expression examined in untreated cells after 5 days or 4 weeks in culture **(Fig.23)**.

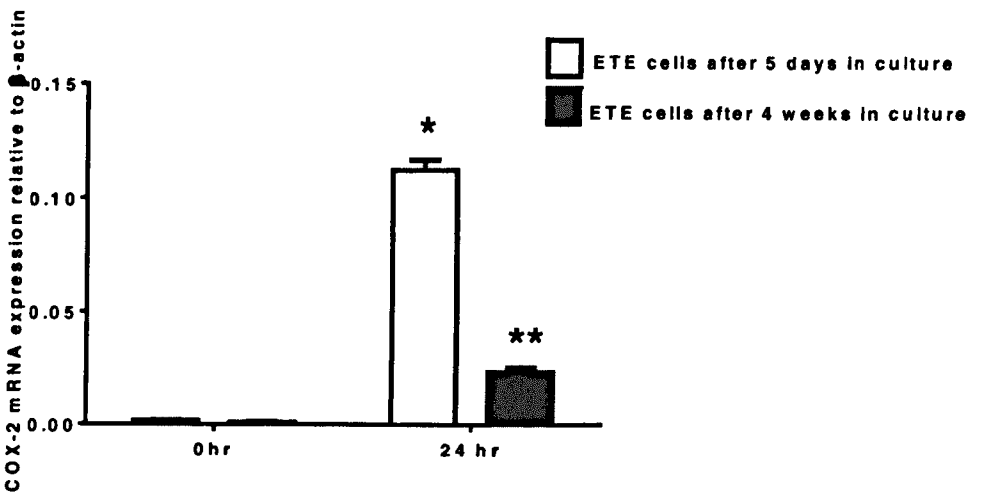

Figure 23: q-PCR graph of COX-2 mRNA expression in ETEC after culture period of 5 days or 4 weeks

The effect of time spent in culture on COX-2 mRNA expression (relative to reference gene β-actin) in untreated ETE cells. Cell samples were taken from each group 24hrs after a fresh media replacement. Primary ETE cells isolated after 2hr trypsin digestion of tracheal epithelial tissue from separate subject to previous experiments. Error bars represent SEM obtained from 3 replicates from a single experiment. A two-way ANOVA was performed to indicate any statistical significance. Expression of COX-2 mRNA was significantly increased (p value < 0.0001) in 5 day cultures (*) after 24hrs compared to time zero and reduced in 4 week cultures (**) compared to 5 days old cultures (p value < 0.001) after 24hrs.

Expression of COX-2 mRNA was significantly increased ($p < 0.0001$) in 5 day cultures (*) after 24hrs in culture compared with time zero. Levels of COX-2 mRNA in 4 week old ETE (**) were significantly reduced ($p< 0.0001$) compared with 5 day old cultures after 24hrs. Reduction in COX-2 mRNA levels at 24hrs in 4 week old ETE cells was considered to be partially caused by reduced growth and activity in fully confluent cells. The

increase in COX-2 mRNA after 24hrs in 5 day old ETE cells was attributed to rapid growth of cells after enzymatic isolation.

Results for both 5 day old and 4 week old ETE cells again indicated that both culture period and the initial enzymatic dissociation of cells from tissue with trypsin were sources of inflammation. Previous attempts to obtain ETE cells with alternative digestion media had been unsuccessful. It was then decided to investigate isolation of ETE cells with reduction of trypsin digestion time. The aim was to collect sufficient numbers of ETE cells but with minimal exposure to digestion media to reduce inflammation.

Effect of tissue digestion period and trypsin concentration

The trypsin concentration and time period of tissue dissociation required to obtain primary epithelial cells was then examined. Trypsin proteolysis of epithelial tissue was inspected every 20 mins under the light microscope for dissociated primary cells. Trypsin action was halted once 10^4/ml of cells was present. Incubation time with trypsin during the initial cell isolation was accordingly reduced from 2hrs to approximately 30-45 mins. This was found to be sufficient to routinely obtain adequate cell numbers, and was the method used in all subsequent experiments where ETE cells were required.

Effect of LPS concentrations on inflammatory expression in primary equine ETE cells

Following the optimisation of trypsin incubation time to obtain ETE cells, it was decided to examine the concentration of LPS required to initiate an inflammatory response. It was thought that the use of 10 µg/ml LPS to

induce inflammation could be excessive, particularly as horses are considered sensitive to the effects of LPS (Moore 2001). Additionally, bronchial epithelial cells act as part of innate respiratory immunity and as such would be highly responsive to LPS treatment.

The effect of three different LPS concentrations on ETE cells isolated with the reduced trypsin incubation time was investigated. Concentrations of 0.1, 10 or 100 μg/ml LPS were used to stimulate primary ETE cells obtained using the reduced trypsin incubation technique. The most noticeable increase in COX-2 mRNA had previously been observed on both control and treated groups at 24hrs, and it was decided to use this time point in future experiments. Expression of mRNA after LPS incubation (relative to β-actin) now included TLR-4, TNF-α, IL-1β and iNOS in addition to COX-2 (**Fig.24).**

Results showed that 0.1μg/ml LPS after 24hrs was significantly (*) increased for ALX (p value 0.004), TLR-4 (p value < 0.0001), TNF-α (p value 0.0003), and IL-1β (p value 0.0012) compared with both untreated samples, and 10 and 100μg/ml LPS concentrations. Apparent increases for iNOS and COX-2 mRNA after 0.1μg/ml LPS incubation were not statistically different. Despite this, levels of mRNA for these genes appeared increased compared with other LPS concentrations.

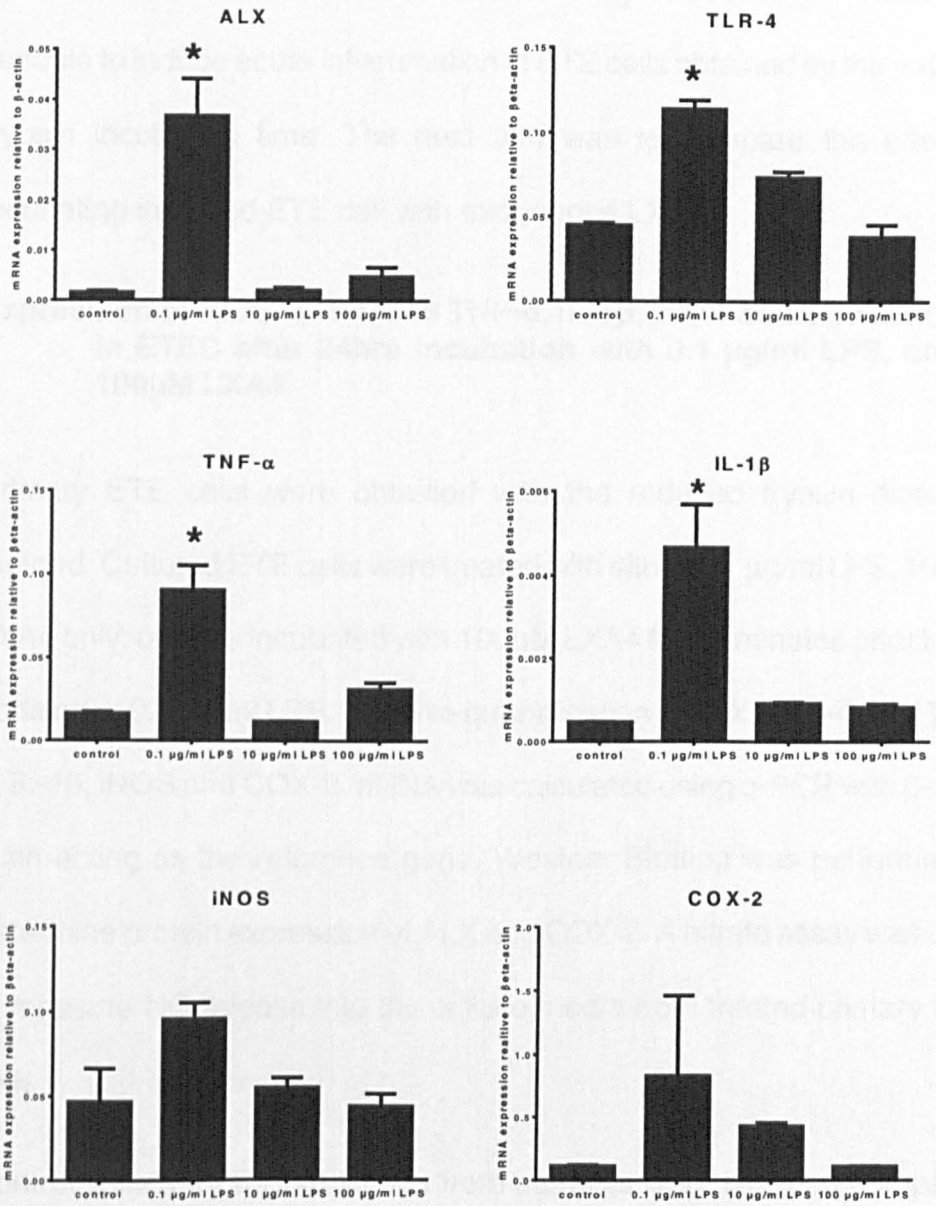

Figure 24: q-PCR graph of mRNA expression in ETE cells after 24hrs incubation with 0.1, 10 and 100 µg/ml LPS concentrations

Expression of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2mRNA mRNA was relative to reference gene β-actin. Primary ETE cells were obtained from 30-45mins digestion of bronchial epithelial tissue from 3 separate subjects to previous experiments. Error bars represent the SEM obtained from 3 sample means from three separate experiments. A one-way ANOVA was performed to indicate any statistical significance. Expression of mRNA statistically significant (*) after 24hr incubation with 0.1µg/ml LPS for ALX, TLR-4, TNF-α, and IL-1β (*p* value 0.04, <0.0001, 0.003 and 0.0012 respectively) compared with controls and 10 and 100µg/ml LPS treatment.

It was concluded that a concentration of 01.µg/ml LPS concentration was suitable to induce acute inflammation in ETE cells obtained by the reduced trypsin incubation time. The next aim was to compare the effect of incubating inflamed ETE cell with exogenous LXA4.

Expression of ALX, TLR-4, and TNF-α, IL-1β, iNOS and COX-2 mRNA in ETEC after 24hrs incubation with 0.1 µg/ml LPS, and/or 100µM LXA4

Primary ETE cells were obtained with the reduced trypsin digestion method. Cultured ETE cells were treated with either 0.1 µg/ml LPS, 100µM LXA4 only, or were incubated with 100µM LXA4 for 15 minutes prior to the addition of 0.1 µg/ml LPS. Relative quantification of ALX, TLR-4, and TNF-α, IL-1β, iNOS and COX-2 mRNA was calculated using q-PCR with β-actin actin acting as the reference gene. Western Blotting was performed to determine protein expression of ALX and COX-2. A nitrate assay was used to measure NO release into the culture media from treated primary ETE cells

Control values for mRNA values from samples were reduced compared with other treatment groups suggesting no inflammation in ETE cells obtained with reduced trypsin digestion. However, treatment with LXA4 or LPS did not appear to have any effect on mRNA expression for any of the selected genes (**Fig.25**).

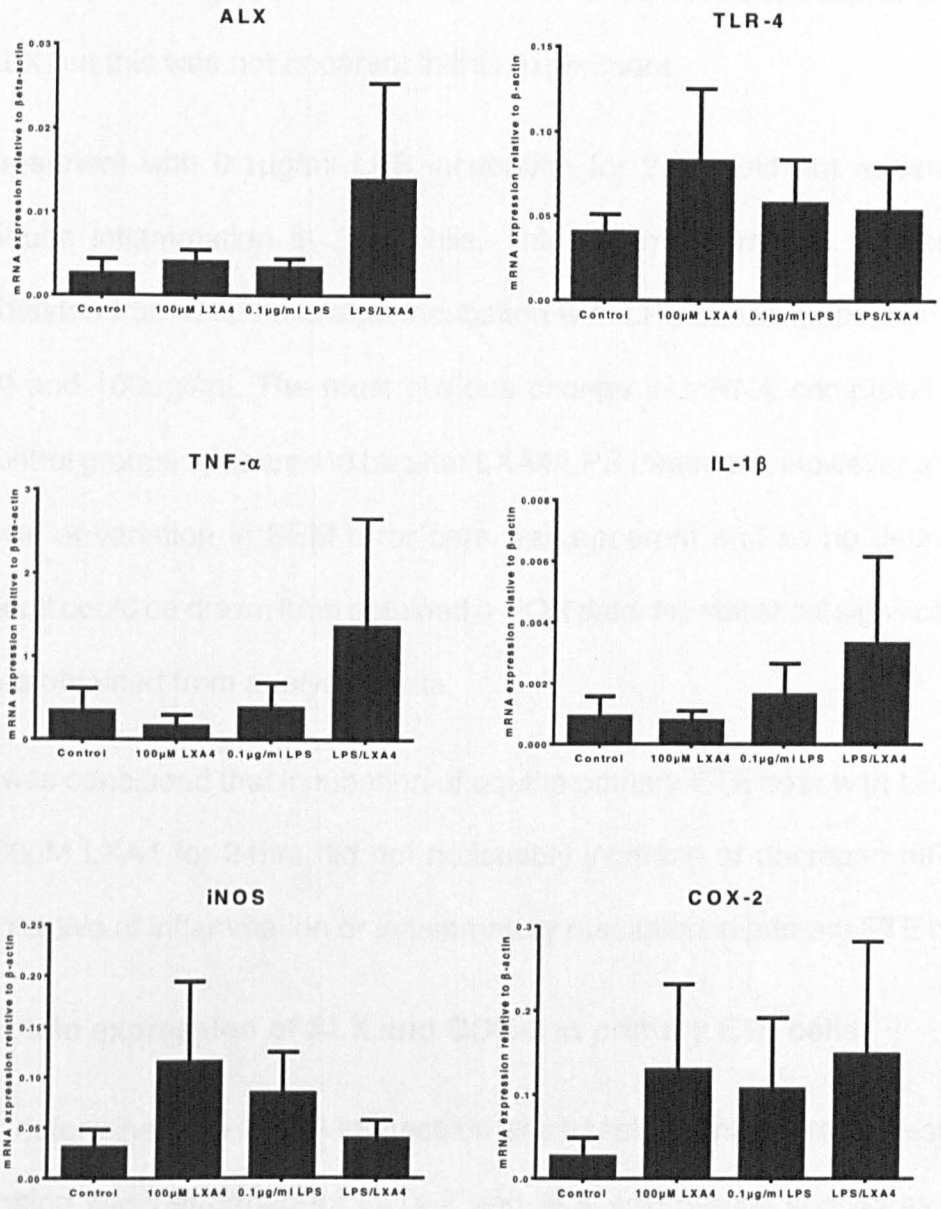

Figure 25: q-PCR graph of mRNA expression in ETEC after 24hrs incubation with 0.1 µg/ml LPS, 100µM LXA4 and/or LXA4

Expression of ALX, TLR-4 TNF, IL-1β, iNOS and COX-2 mRNA was relative to reference gene β-actin.ETE cells obtained from 3 separate subjects to previous experiments. Error bars represent SEM obtained from 3 sample means from three separate experiments. A one-way ANOVA was performed to indicate any statistical significance.

Incubation of ETE cells with 100µM LXA4 did not increase ALX mRNA expression which contradicts findings in other lipoxin studies (Bonnans *et*

al 2006). Binding of LXA4 to receptor ALX should increase expression of ALX but this was not apparent in this experiment.

Treatment with 0.1μg/ml LPS incubation for 24hrs did not apparently induce inflammation in ETE cells. This again contradicts the results obtained from ETE cells after incubation with LPS concentrations of 0.1, 10 and 100μg//ml. The most obvious change in mRNA compared with control groups appeared to be after LXA4/LPS treatment. However a high level of variation in SEM error bars was apparent and so no definitive result could be drawn from obtained q-PCR data. No statistical significance was obtained from analysed data.

It was concluded that incubation of equine primary ETE cells with LPS or 100μM LXA4 for 24hrs did not noticeably increase or decrease mRNA indicative of inflammation or inflammatory resolution in primary ETE cells

Protein expression of ALX and COX-2 in primary ETE cells

To determine if the mRNA expression was translated into protein, Western blotting was performed for COX-2 and ALX with β-actin serving as the reference protein. ETE cells were obtained from reduced trypsin digestion of tissue from a separate horse to previous experiments. Primary ETE cells were incubated with either 0.1 μg/mL of LPS, 100 μM LXA4, or pre-treated with 100μM of LXA4 prior to LPS incubation. Protein bands for ALX, COX-2 and β-actin were observed at approximately 30 kDa, 60-80 kDa and 40 kDa respectively which was the predicted molecular weight (Chapter 2) for these antibodies (**Fig.26**).

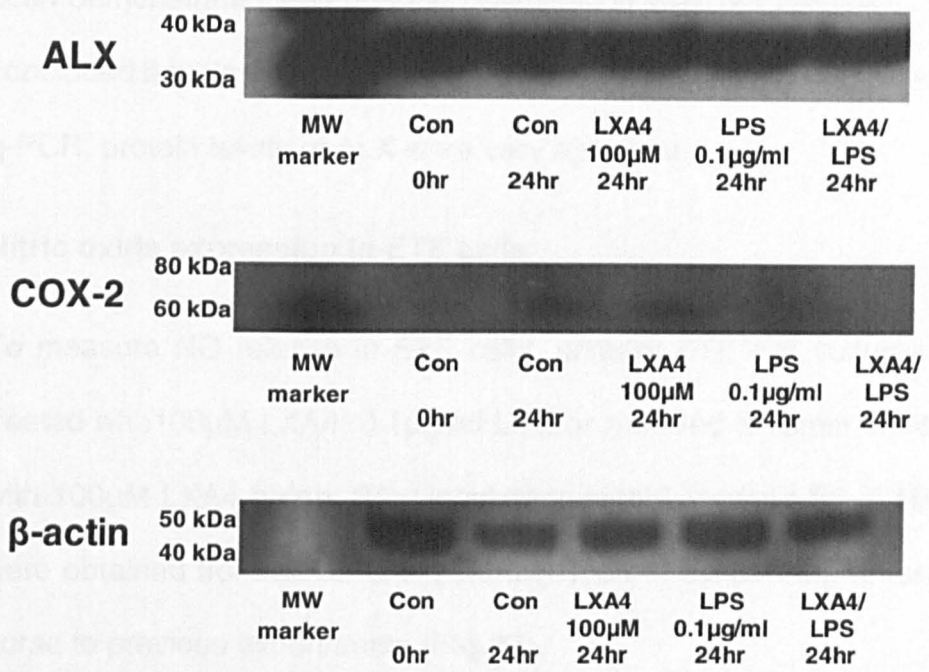

Figure 26: Western blot image of protein expression of β-actin, COX-2 and ALX in primary ETE cells after 24hr incubation with 0.1µg/ml LPS and/or 100µM LXA4

ETE cells were treated with 0.1µg/ml LPS, 100µM LXA4, or pre-treated for 15 minutes with 100µM LXA4 before LPS incubation. Primary ETE cells were obtained from 30-45mins trypsin digestion of bronchial epithelial tissue from a separate tissue subject to previous experiments.

Western blotting revealed that protein expression of ALX in all treatment groups at 0 and 24hrs was very strongly evident. This suggested that although mRNA for ALX had not been significantly expressed, protein levels were detectable. In contrast, COX-2 bands at the same time point were weakly expressed, and absent in controls and after LPS/LXA4 incubation

Results for COX-2 potentially suggested although protein in control and LXA4/LPS samples was undetected, this may have been caused by primary antibody inefficiency, rather than an absence of COX-2. The possibility of antibody inefficiency from poor technique was discounted as the strong and robust bands observed in the house-keeping antibody β-

actin demonstrated that method optimisation was not required. It was concluded that despite the absence of ALX mRNA in results obtained from q-PCR, protein levels of ALX were very apparent.

Nitric oxide expression in ETE cells

To measure NO release in ETE cells, primary ETE cell cultures were treated with100μM LXA4, 0.1μg/ml LPS or received a 15min incubation with 100μM LXA4 before 24hr incubation with 0.1μg/ml LPS. ETE cells were obtained from reduced trypsin digestion of tissue from a separate horse to previous experiments (**Fig.27**).

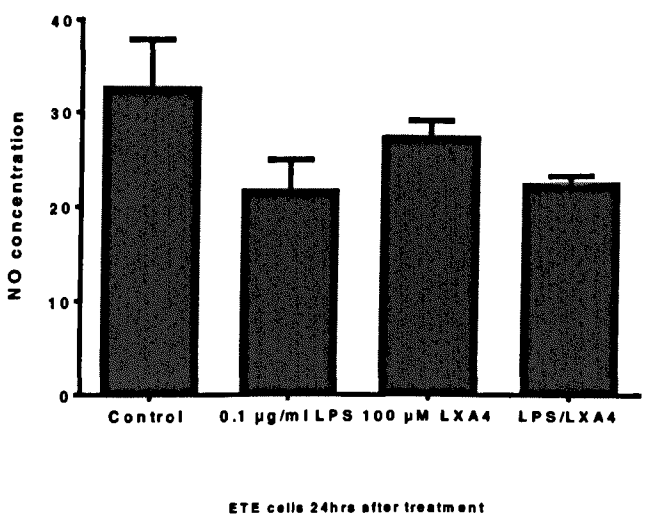

Figure 27: Quantative graph representing NO expression in primary ETE cells after incubation with 0.1μg/ml LPS, 100μM LXA4 and/or LXA4/LPS concentrations. NO expression measured by fluorescent intensity (relative units to nitrite/nitrate standard curve)

Media supernatant was assayed by fluorometric analysis for NO release from ETE cells 24hrs after treatment. Primary ETE cells were obtained from 30-45mins trypsin digestion of bronchial epithelial tissue from a separate tissue subject to previous experiments. Error bars represent SEM obtained from 3 replicates from a single experiment. A one-way ANOVA was performed to indicate any statistical significance.

Supernatant from the treated cells was assayed for NO expression by fluorometric analysis. Nitrate analysis did not show any discernible difference between ETE samples after treatment with 100μM LXA4, 0.1μg/ml LPS or the combined LPS/LXA4 incubation. Analysis of results with a one-way ANOVA did not reveal any statistical significance in data. It was concluded that LPS and LXA4 treatment of ETE cells did not have any effect on NO expression

4.5 Discussion

After an *in vitro* model of ETE cells had been successfully established and characterised (Chapter 3), the next stage was to establish a suitable time point and LPS concentration to induce inflammation in cultured cells from this model. Adjustments to the method to obtain trypsin-dissociated cells were required as both end-point and q-PCR analysis detected COX-2 mRNA expression in untreated cell samples. Potential areas of contamination which might have been responsible for this were examined. Consideration was given to tissue collection (aseptic technique, tissue transport), preparation of tissue, culture conditions, RNA extraction and cDNA synthesis.

One potential difference to the original methodology was the transport of tissue in cell culture grade PBS rather than Hanks Buffered Salt Solution (HBSS). The use of HBSS may have offered a very minor improvement in cell osmolarity due to the enhanced salt ion formulation. However, optimisation of all aspects of the methodology did not eliminate the underlying acute inflammation as measured by COX-2 mRNA and detected in untreated cells by end-point PCR.

Quantifying the response of ETE cells to LPS with end-point PCR was initially unsuccessful. To increase the level of mRNA detection, samples were subsequently quantified by real time PCR. ETE cells were incubated with 10μg/ml LPS concentration for 0, 4 and 24hrs. Significant reduction of COX-2 mRNA was observed in both control and LPS treated samples (*p* value 0.0001) at time zero, and in control samples at 4hrs (*p* value <0.0001). It was concluded that any apparent subsequent increase in COX-2 mRNA representative of inflammation was unrelated to LPS treatment.

Incubation with carprofen did not significantly decrease COX-2 mRNA. Carprofen is a COX-2 inhibitor which is frequently used for chronic inflammation associated with osteoarthritis (Goodrich & Nixon 2006). Carprofen is also reported to inhibit LPS-induced iNOS expression in ovine macrophages (Bryant *et al* 2003). Conversely, carprofen did not inhibit LPS-induced COX-2 activity *in vitro* with samples of whole blood from horses (Brideau *et al* 2001). It may be that carprofen is unsuitable or only partly effective at controlling inflammation in equine ETE cells *in vitro*. It was concluded that neither anti-inflammatory nor inflammatory stimulation of ETE cells obtained after 2hrs trypsin-dissociation significantly altered COX-2 mRNA expression at 4 and 24hrs.

The effect of culture conditions on ETE cells obtained from 2hr trypsin digestion was examined. It was established that COX-2 mRNA expression was significantly increased ($p< 0.0001$) in 5 day ETE cells obtained after 2hr trypsin dissociation after 24hrs in culture. The inflammation observed in ETE cells which was attributed to the effects of trypsin digestion of

tissue. Such inflammation could have been eliminated by passaging cells, an approach not attempted during ETE cell culture. Passaging ETE cells obtained by trypsin dissociation was described in the original method (Shibeshi *et al* 2008). This technique required the use of dispase II and collagen coated tissue culture flasks for cell adherence. The reliance of dispase II-passaged ETE cells on collagen coated culture plates for adherence indicated that essential cell viability and function was potentially compromised. Additionally no inflammatory profile was described in ETE cells passaged with dispase II. It was concluded that further manipulation of ETE cells would not necessarily eliminate any inflammation in primary cells resulting from the initial trypsin digestion of tissue.

Finally, the effect of trypsin on dissociated primary cells was considered. Trypsin has been linked to TNF-α and IL-1β mRNA release in rat peritoneal macrophages as early as 30 mins after exposure and reaching peak levels at 16hrs (Lundberg 2000). Reduction of the incubation times with trypsin but not enzyme concentration successfully produced adequate cell numbers of good viability.

After optimisation of the trypsin digestion time, the effect of LPS concentrations on ETE cells was examined. In contrast to findings in the initial work on primary ETE cells, LPS-induced inflammation in other studies of epithelial and respiratory cells with a LPS concentration of 10μg/ml has been successful (Wu *et al* 2002, Hostanka *et al* 2011, Xu *et al* 2011, Miao et al 2012). It was considered that the LPS concentration of 10μg/ml was excessive for equine ETE cells, and effectively induced cell apoptosis. ETE cells were therefore incubated with 0.1, 10 and 100μg/ml

LPS concentrations. ALX, TLR-4, TNF-α, and IL-1β mRNA expression were significantly increased (*p* values < 0.0005) after 0.1μg/ml LPS after 24hrs. Although not statistically significant, levels of mRNA for iNOS and COX-2 also appeared most increased after 0.1μg/ml LPS. An excessive concentration of LPS affecting ETE cell viability would be in agreement with an investigation of LPS effects on *in vitro* rat cell cultures. Primary rat tracheal epithelial cells were cultured with 10μg/ml of LPS for 18 hours. Morphological changes in cultured cells, altered rates of proliferation, and decreased cell density were detected. Cellular alterations were attributed to the adverse reaction of cells to LPS (Freitag *et al* 1996).

The selected LPS concentration of 0.1μg/ml LPS was used in subsequent treatment of ETE cells with LXA4 and LPS to determine the potential for inflammatory resolution. Overall, treatment with LPS and LXA4 did not apparently increase or inhibit mRNA expression in any of the selected genes. It initially appeared that LXA4 incubation marginally reduced TNF-α and IL-1β mRNA. Unusually, primary ETE cells cultured with exogenous LXA4 did not show an increase in ALX mRNA expression as would be expected. This is in contrast to a previous report that showed that ALX receptor activity is increased following binding to its ligand LXA4 (Serhan 2005). Lack of ALX expression in our studies may be caused by several reasons. A simple explanation is that the receptor binding is relatively brief or occurs prior to our chosen time point of 24hrs. Peak expression of ALX has been observed in human bronchial epithelial cells 2 hours after acid injury to cells. Expression of ALX mRNA steadily decreased thereafter,

with levels reduced to almost half at 24 and 72 hours (Bonnans *et al* 2006).

Inconclusive results after LXA4 studies were potentially caused by the previous use of COX-2 to signify inflammation at the chosen time point of 24hrs. COX-2 expression unrelated to inflammatory episodes is indicated in other studies, with levels deemed to be cell-specific and unpredictable (Ermert *et al* 2000). COX-2 mRNA has been detected in baseline conditions in rat bronchial epithelial cells (Ermert *et al* 2011). COX-2 mRNA and protein is also commonly expressed in human bronchial epithelial cells and BEAS-2B cells in culture without inflammatory stimulation (Watkins *et al* 1999, Pierzchalska *et al* 2007). Ermert *et al* 2011). Minimal COX-2 mRNA expression has been found in bronchial biopsy specimens from non-asthmatics (0.6%) compared with asthmatics (4.6%) (Redington *et al* 2001). This indicates a potential for irregular expression of COX-2 that is subject-specific and differs according to minor changes in tissue source or the health of each individual. It also suggests that COX-2 is not as reliable a marker of inflammation as previously thought.

A time point of 24hrs to detect inflammation had been considered suitable based on previous experiments with COX-2 mRNA. Comparison of findings at 24hrs in ETE cells after LPS and LXA4 investigations indicate that earlier time points are necessary to accurately quantify peak levels of mRNA expression. Early COX-2 expression has been demonstrated in other equine cells associated with acute inflammation. Equine leukocytes expressed COX-2 mRNA at 30 minutes and at 4hrs after 10 µg/ml LPS

(Eckert *et al* 2007). Additional increases of both mRNA and protein were observed within 1hr after 10μg/ml LPS treatment (Ermert *et al* 2011). Expression of molecules such as TNF-α and IL-1β may also occur also earlier than at 24hrs. Detection of TNF-α and COX-2 was observed in equine monocytes within 1hr of treatment with 100pg/ml LPS but expression had declined to baseline at 20hrs (Sun *et al* 2010). Use of a single time point at 24hrs was therefore an important limiting factor in assessing inflammation and inflammatory resolution. Our study performed some pilot trials on COX-2 mRNA expression at 1 and 4 hours (data not shown), but there was very little difference in mRNA expression levels. Ideally, samples should have been taken at much earlier time points; for example 15 mins, 30 mins 1hr, 4hrs and 24hrs. These particular points were beyond the time and resources of this study.

Detection of protein with Western blotting after LXA4 studies showed that signal for ALX protein was highly abundant and signal for COX-2 protein very weak. This may have been due to insufficient protein in the sample, or the antibody concentration being too low. However, protein samples were quantified as sufficient before use (Chapter 2) and the strong signal detected in ALX and loading control β-actin discounted this possibility.

It was concluded that absence of bands in COX-2 was more likely to result from a poorly performing antibody, rather than low protein levels. The primary antibodies were selected for their reported performance in several species and applications. No cross-reactivity for use in equine tissue in either primary antibody was described on the data sheet. However, BLAST analysis of the amino acid sequence for each primary antibody suggested

they would be suitable to detect protein levels in equine cells. Steps to optimise the COX-2 primary antibody should have attempted, such as performing a series of experimental dilutions to determine an optimal antibody concentration. Additionally, efforts should have been made to test the primary antibody on the suggested positive control, the mouse macrophage cell line Raw264.7. Finally, it should be considered that that particular chosen COX-2 primary antibody was suspect or simply unsuitable for use in equine tissue or Western blotting. The validity of data for protein expression could also have been improved if experimental repeats had been performed.

Similarly, nitrate analysis by fluorometric detection of ETE cells would produce more reliable data if further repeats were carried out. Lack of observable differences in NO expression in ETE cells may be caused by sampling at the wrong time. Expression of iNOS mRNA is seen as early as 6hrs (Hao *et al* 2011), with levels of NO observed in human bronchial epithelial cells after 12 and 24hrs stimulation with TNF-α (Robbins *et al* 1994), and in murine epithelial cells at 24hrs (Robbins *et al* 1994). Expression of NO can be affected by culture conditions and growth factors in media (Asano *et al* 1994). Accurate quantification can therefore be challenging.

Results from experiments examining COX-2 mRNA expression and determining time points were based on samples obtained from a different horse for each experiment. Similarly, LXA4 data resulted from tissue obtained from three different horses. Both factors provide considerable scope for variation in cell expression due to potential differences in each

subject's health and history. Repeated testing from a small number of the same horses would enable to the development of an individual immune profile for each horse. This could then determine if similar cellular responses between each horse could be observed during induced inflammation or inflammatory resolution *in vitro*. Unusual or dissimilar data obtained from experiments could then be attributed to either a particular individual or a single experiment. This should eliminate much of the variation observed in collected data, particularly the LXA4 experiments.

It was concluded that use of a different horse for each experiment was the strongest factor in producing comparable and reliable data.

4.6 Conclusion

An *in vitro* respiratory epithelial cell model is suitable for use in investigations of airway inflammation and potential inflammatory resolution. Treatment of ETE cells with LPS induces some evidence of inflammation but LXA4 incubation does not cause observable inflammatory resolution. Alterations to the chosen time points are required, as is assessment of COX-2 as a marker of inflammation, and the concentration of LXA4 used to upregulate ALX. Such adjustments may then improve investigations of LXA4 into airway inflammation in horses.

Chapter 5: Effect of LXA4 incubation on acute inflammation in primary equine airway smooth muscle cells

5.1 Introduction

Remodelling of ASM after chronic inflammatory exacerbations initiates an increase in the layer of trachealis muscle in both human and horse (Robinson *et al* 1996). Factors contributing to ASM increase include cell hyperplasia or hypertrophy (Wright *et al* 2012), failure of cell apoptosis (Ding *et al* 2008) and cell migration (Halwani *et al* 2011).

An increase ASM in human and horses significantly contributes to airway hyper-responsiveness and is relative to disease advancement (James *et al* 2009). ASM increase is also highly apparent in people affected by severe or refractory asthma (Macedo *et al* 2009, Tillie-LeBlond *et al* 2008). Prevention of ASM increase or reversal of such remodelling changes is a focus of asthma and RAO treatment.

5.2 Aims and objectives

- Establish the response of *in vitro* ASM cells to incubation with LPS, the same inflammatory mediator used to incubate primary ETE cells
- Compare the LPS-induced response of ASM cells to treatment with LXA4 treatment, to investigate potential inflammatory resolution.

To achieve these aims, the *in vitro* model of primary ASM cells established in Chapter 3 was incubated with different concentrations of LPS at varying

time points. This was to determine a suitable time point and LPS concentration to induce inflammation.

Inflammatory resolution was examined by comparing the selected time point and LPS dose with LXA4 or a combination of both LPS and LXA4. The response of ASM cells to treatment was analysed by quantification of mRNA and detection of protein expression.

5.3 Methods

Primary equine ASM cells were cultured from explants of trachealis muscle and maintained in serum-supplemented DMEM media until 70-75% confluent. ASM cells obtained from explants were not passaged before experimental use.

Primary ASM cultures were treated with LPS concentrations of 0.1, 10 and 100μg/ml to determine a suitable level of LPS to induce acute inflammation. Expression of COX-2 mRNA was used to signify inflammation at the chosen time points of 12, 24 and 72hrs based on similar papers (Belvisi *et al* 1997, Pang & Knox 1997, Chen& Khalil 2006)

The response of ASM cells to inflammatory stimulus was compared with the effect of LXA4 treatment. ASM cells were incubated with LPS, LXA4 or pre-treated with LXA4 for 15mins before incubation with LPS. Control samples for ASM cells did not receive either LXA4 or LPS treatment. The same methods used in Chapter 4 were used here with details explained in Chapter 2.

5.4 Results

ASM expression of COX-2 mRNA at 12, 24 and 72hrs after incubation with 0.1, 10 and 100µg/ml LPS concentrations

A time and concentration study was performed to determine the response of ASM cells to LPS incubation. Concentrations of 0.1 and 10µg/ml LPS induced the most noticeable levels of COX-2 expression at 72 hours in ASM cells compared with all other samples (**Fig.28)**

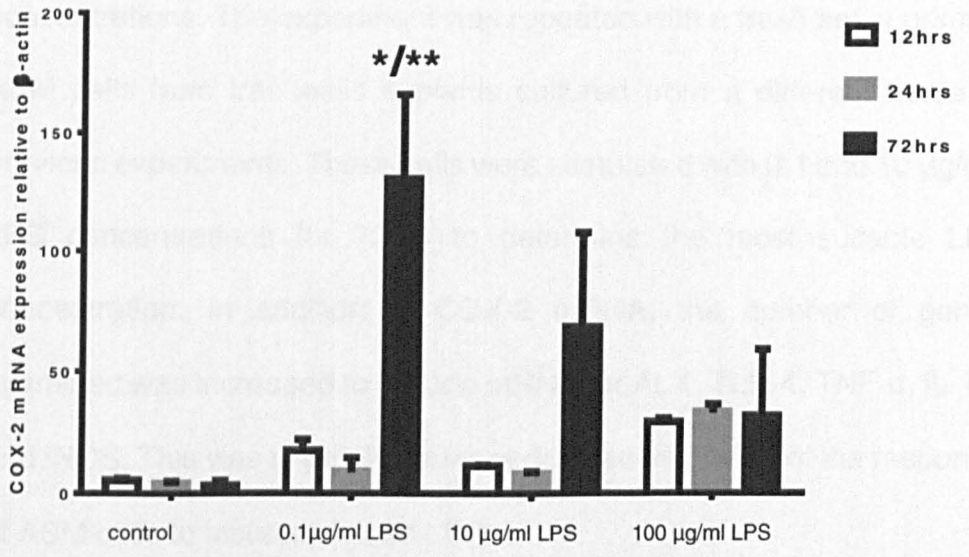

Figure 28: q-PCR graph of COX-2 mRNA expression in primary ASM cells after time and concentration course of LPS

COX-2 mRNA (relative to reference gene β-actin) was quantified at 12, 24 and 72hrs after treatment with 0.1, 10 and 100µg/ml LPS concentrations. ASM cells were obtained from a separate tissue subject to previous experiments. Error bars represent SEM obtained from 3 replicates for each sample from a single experiment. A two-way ANOVA was performed to indicate any statistical significance. Expression of COX-2 mRNA was significantly increased after 72hrs (*) of 0.1µg/ml LPS (**) compared with controls (*p* value 0.0115) and all other time points (*p* value 0.0015).

Data analysis with a two-way ANOVA revealed 0.1µg/ml LPS (**) significantly increased COX-2 mRNA levels compared to control samples (*p* value 0.0115) but not to 10 or 100µg/ml LPS concentrations. Expression of COX-2 after 72hrs (*) of 0.1µg/ml LPS was also significantly increased compared to 12 and 24hrs (*p* value 0.0015), suggesting this was a suitable

time point to examine inflammation in ASM cells. In contrast to ETE cells, no noticeable expression of COX-2 mRNA in control samples was observed at the chosen time points of 12, 24 and 72hrs. This indicated that ASM cells cultured from airway smooth muscle explants were free from detectable inflammation prior to use in experiments.

Although not statistically significant, COX-2 mRNA mean values for 10μg/ml LPS were closely similar to those from 0.1μg/ml LPS concentrations. The experiment was repeated with a fresh set of primary ASM cells from trachealis explants cultured from a different horse to previous experiments. These cells were stimulated with 0.1 and 10 μg/mL LPS concentrations for 72hrs to determine the most suitable LPS concentration. In addition to COX-2 mRNA, the number of genes examined was increased to include mRNA for ALX, TLR-4, TNF-α, IL-1β, and iNOS. This was to provide a more detailed indication of the response of ASM cells to incubation with LPS.

Expression of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA in primary ASM cells after 0.1 and 10 μg/mL LPS concentrations.

Results showed that mRNA levels after incubation with 0.1 and 10 μg/mL LPS concentrations were still very similar (**Fig.29**).

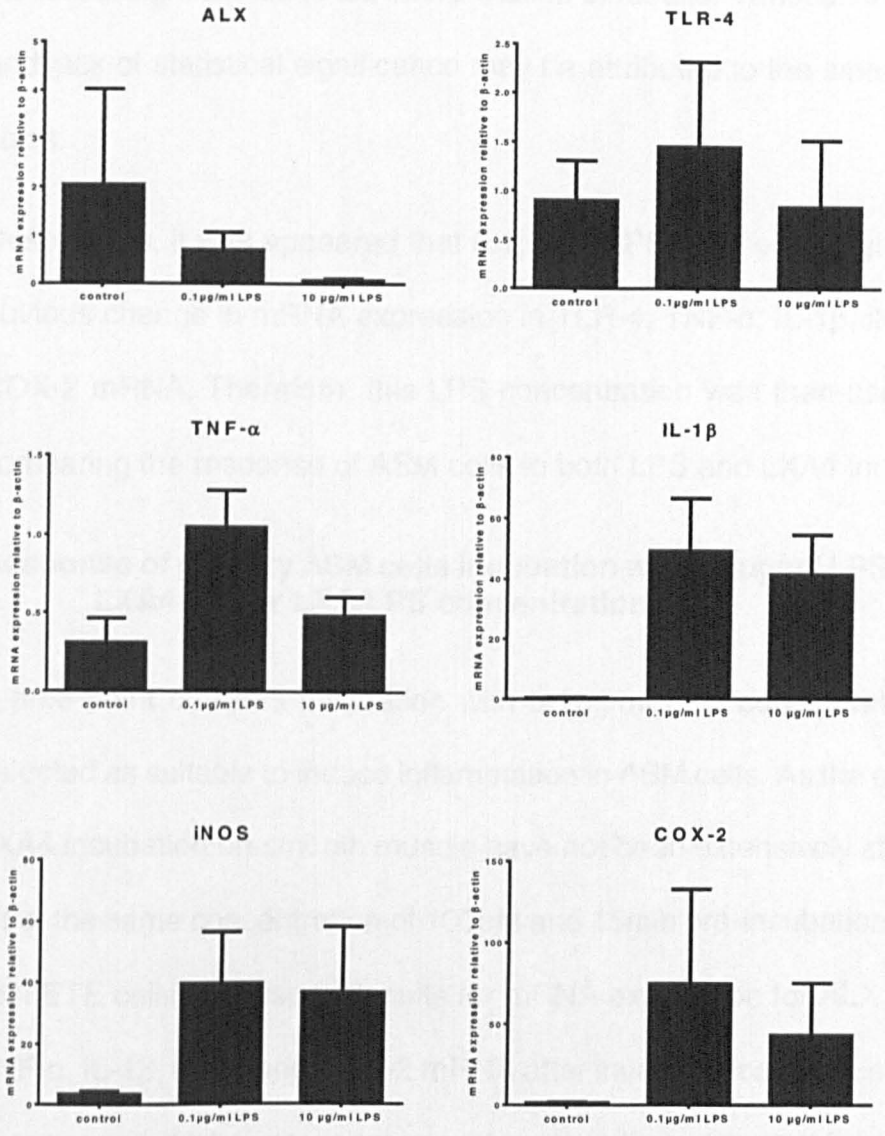

Figure 29: q-PCR graph of mRNA expression in primary ASM cells after incubation with 0.1, and 10μg/ml LPS concentrations

Expression of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA (relative to reference gene β-actin) was quantified after 72hrs treatment. ASM cells were obtained from trachealis muscle explants from separate tissue subject to previous experiments. Error bars represents SEM obtained from 3 replicates for each sample from a single experiment. A one-way ANOVA was performed to indicate any statistical significance.

Error bars variation was particularly noticeable for ALX mRNA expression. LPS incubation appeared to decrease ALX mRNA compared with control values. One way ANOVA analysis of mRNA data did not reveal any

statistical significance to aid interpretation of results. Variation in the data and lack of statistical significance may be attributed to the small sample sizes.

Despite this, it was appeared that 0.1μg/ml LPS induced a slightly more obvious change in mRNA expression in TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA. Therefore, this LPS concentration was then used when comparing the response of ASM cells to both LPS and LXA4 incubation.

Response of primary ASM cells incubation with 0.1μg/ml LPS, 100μM LXA4 and/or LXA/LPS concentrations.

A time point of 72hrs incubation with 0.1μg/ml LPS concentration was selected as suitable to induce inflammation in ASM cells. As the effects of LXA4 incubation on smooth muscle have not been extensively studied *in vitro*, the same concentration of 100μM and 15min pre-incubation time as with ETE cells was used. Results for mRNA expression for ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA after investigation of the effects of exogenous LXA4 were variable and inconsistent at both 24 and 72hrs (**Fig.30, 31**).

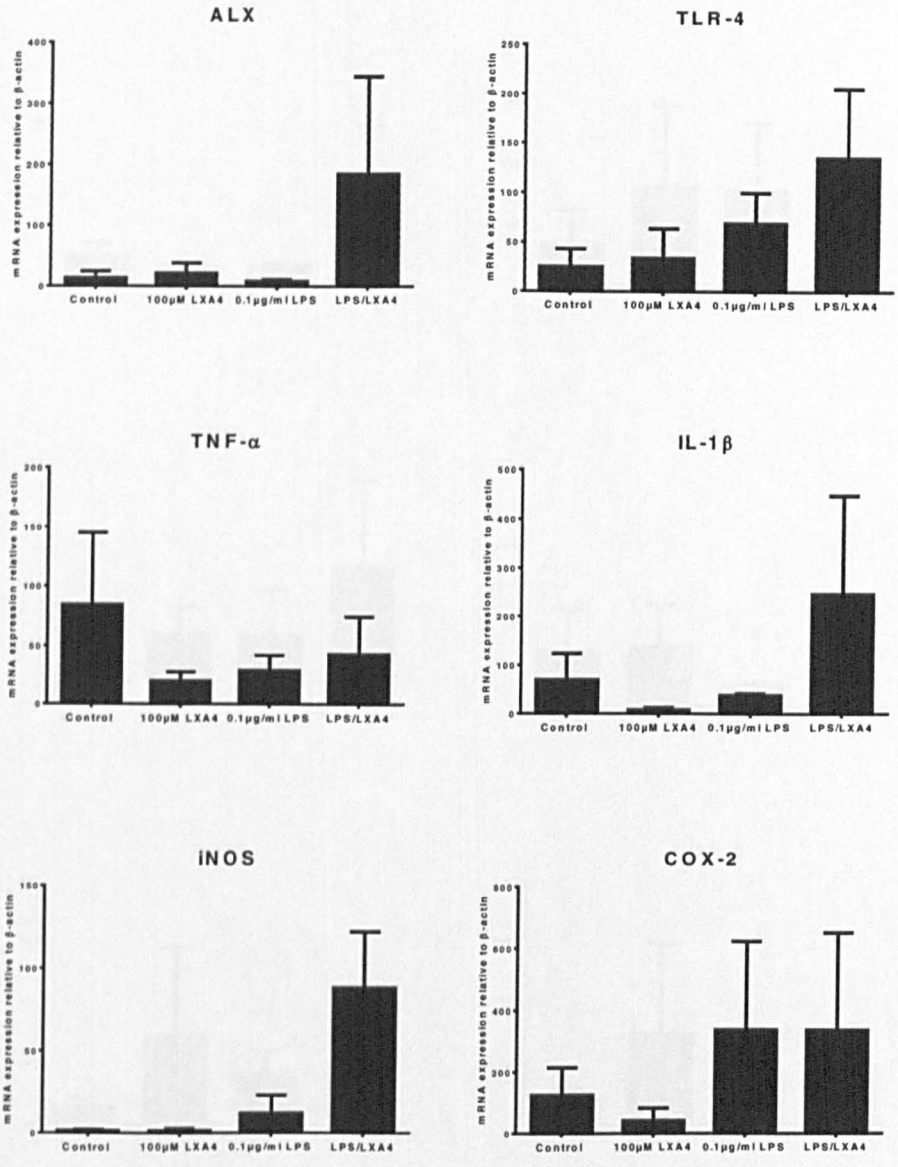

Figure 30: q-PCR graph of mRNA expression in primary ASM cells after 24hrs incubation with 0.1μg/ml LPS, 100μM LXA4 and/or LXA/LPS concentrations.

Expression of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA (relative to reference gene β-actin) was quantified in primary ASM cells after 24hrs treatment. Primary equine ASM cells were obtained from trachealis muscle explants from 3 separate tissue subjects to previous experiments. Error bars represents SEM obtained from 3 sample means from three separate experiments. A one-way ANOVA was performed to indicate any statistical significance

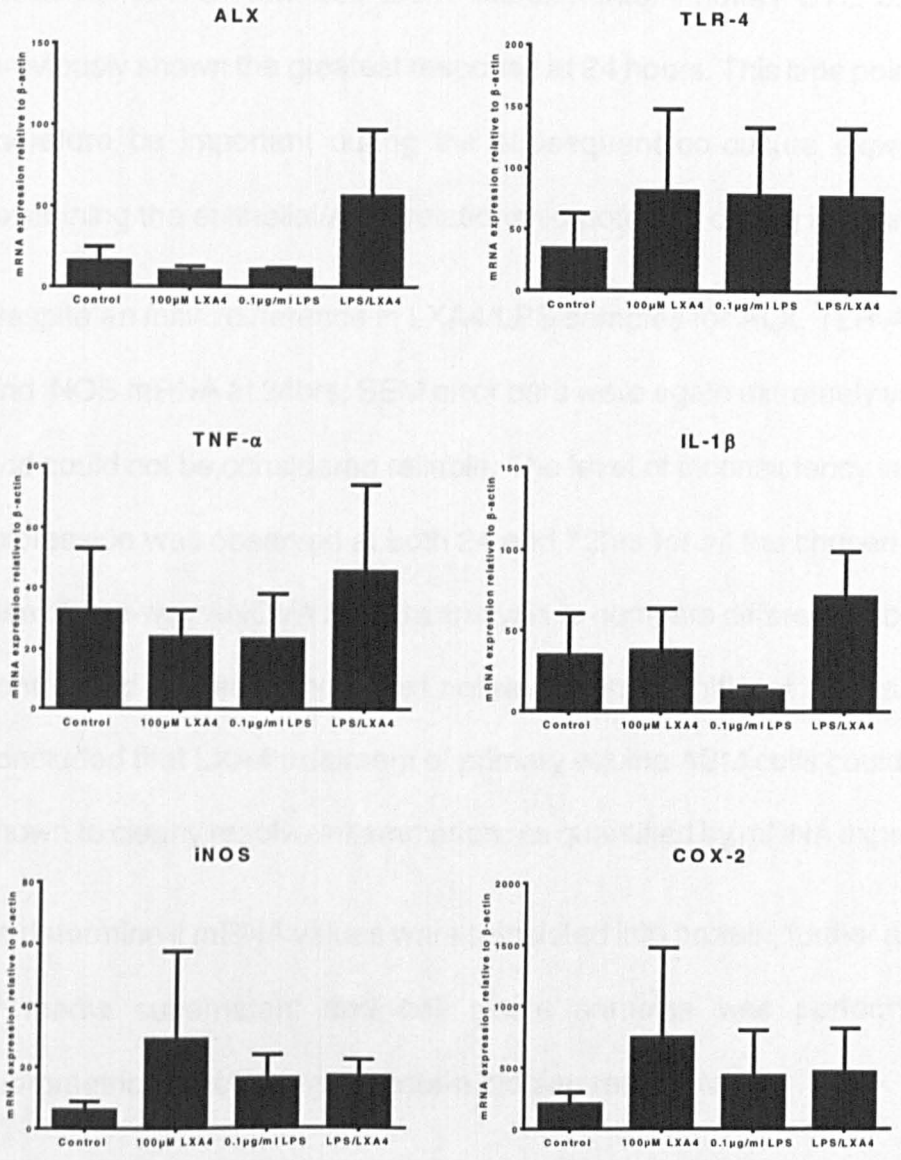

Figure 31: q-PCR graph of mRNA expression in primary ASM cells after 72hrs incubation with 0.1μg/ml LPS, 100μM LXA4 and/or LXA/LPS concentrations.

Expression of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA (relative to reference gene β-actin) was quantified in primary ASM cells after 72hrs treatment. Primary equine ASM cells were obtained from the same tissue subjects as that at 24hrs. Error bars represents SEM obtained from 3 sample means from three separate experiments. A one-way ANOVA was performed to indicate any statistical significance.

Values for treatment samples were frequently similar to control groups for all genes, suggesting that incubation with LXA4 or LPS had little or no effect on ASM cells. Although a negative result was shown for COX-2 mRNA expression after 24hr incubation of ASM cells this time point was

included for the ASM cell LXA4 experiments. Primary ETE cells had previously shown the greatest response at 24 hours. This time point would therefore be important during the subsequent co-culture experiments examining the epithelial/ASM relationship potential during inflammation.

Despite an initial difference in LXA4/LPS samples for ALX, TLR-4, IL-1β, and iNOS mRNA at 24hrs, SEM error bars were again extremely variable, and could not be considered reliable. The level of inconsistency in mRNA expression was observed at both 24 and 72hrs for all the chosen genes. Use of one-way ANOVA for data analysis to compare differences between control and treated samples did not reveal any significant results. It was concluded that LXA4 treatment of primary equine ASM cells could not be shown to clearly resolve inflammation, as quantified by mRNA expression.

To determine if mRNA values were translated into protein, further analysis of media supernatant and cell lysate samples was performed by fluorometric detection and Western blotting respectively.

Protein levels of COX-2 and ALX in ASM cells after 24 and 72hrs treatment with LPS and/or LXA4

Western Blotting was performed on ASM cells after 24 and 72hrs incubation with either 0.1, 10 100 μg/mL of LPS, 100 μM LXA4, or LPS/LXA4 incubation. The three different LPS concentrations were included as previous mRNA expression with 0.1 and 10 μg/mL of LPS had been closely similar. The proteins of interest were ALX and COX-2, with β-actin serving as the reference gene. Protein bands for ALX, COX-2 and β-actin were observed at approximately 30 kDa, 60-80 kDa and 40 kDa

respectively (Chapter 2) which was the predicted molecular weight for these antibodies (**Fig.32).**

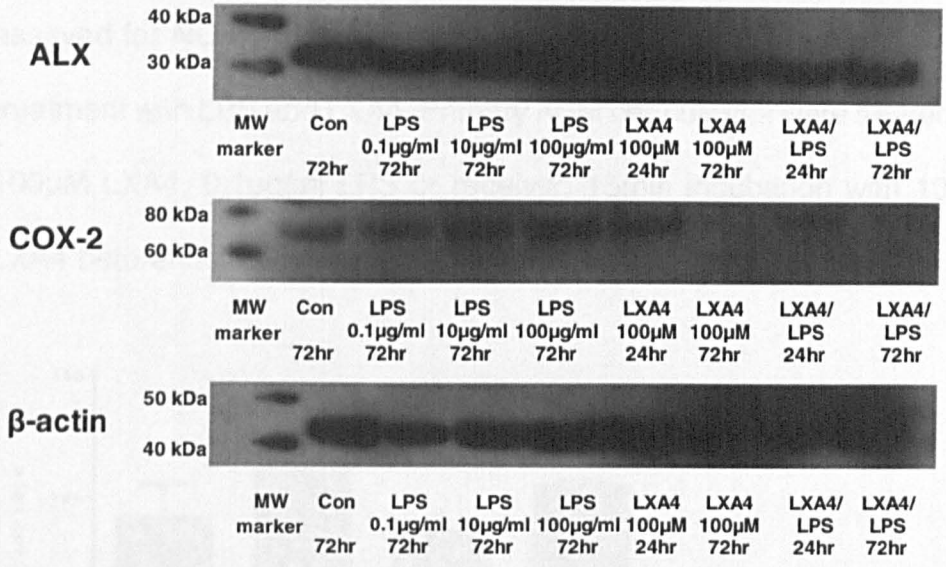

Figure 32: Western blot image of protein expression of β-actin, COX-2 and ALX in primary ASM cells after incubation with 0.1, 10 and 100µg/ml LPS and/or 100µM LXA4 for 24 and 72hrs.

ASM cells were treated with 0.1µg, 10 and 100µg/ml LPS, 100µM LXA4, or pre-treated for 15 minutes with 100µM LXA4 before LPS incubation. Primary equine ASM cells were obtained from trachealis muscle explants from separate tissue subject to previous experiments.

As previously seen in ETE cells, the antibody for ALX protein produced a very strong band in all treatment groups. Results for protein expression of COX-2 showed bands that were again weaker than that of ALX. However, detected bands were stronger than those observed in ETE cell samples. A protein band was present for COX-2 control sample for ASM cells at 72hrs, suggesting a level of inflammation not caused by LPS incubation. In contrast, bands corresponding to COX-2 protein expression after LXA4 and LXA4/LPS treatment were very faint. This indicates that although LXA4 incubation had no discernible effect on mRNA expression, it could have modestly reduced COX-2 protein levels at 72hrs.

Nitric oxide release from ASM cells after 24 and 72hrs treatment with LPS and/or LXA4

In addition to Western blot detection of protein, cultured ASM cells were assayed for NO release in cell supernatant samples 24 and 72hrs after treatment with LPS and LXA4. Primary ASM cell cultures were treated with 100μM LXA4, 0.1μg/ml LPS or received 15min incubation with 100μM LXA4 before incubation with 0.1μg/ml LPS (**Fig.33**).

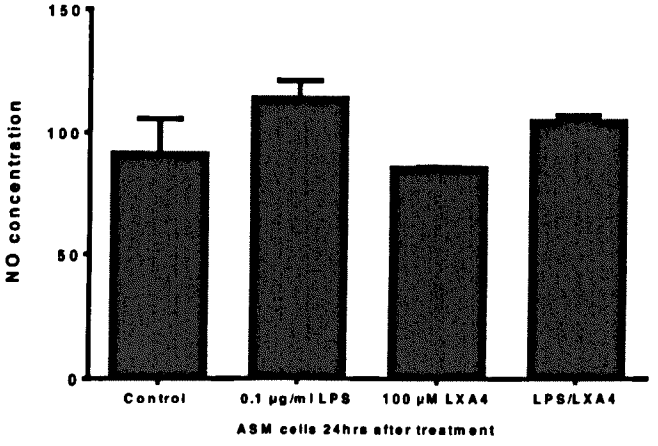

Figure 33: Quantative graph of fluorometric detection of NO expression in primary ASM cell supernatant after incubation with 0.1/ml LPS and/or 100μM LXA4 concentration over 24hrs. NO expression measured by fluorescent intensity (relative units to nitrite/nitrate standard curve)

Primary ASM cells were treated with 0.1μg/ml LPS, 100μM LXA4, or pre-treated for 15 minutes with 100μM LXA4 before LPS incubation. Primary equine ASM cells were obtained from trachealis muscle explants from separate tissue subject to previous experiments. Each error bar represent a group mean (SEM) obtained from 3 replicates means from a single experiment. A one-way ANOVA was performed to indicate any statistical significance

LXA4 treatment of ASM cells after 24hrs appeared to very marginally reduce NO expression compared with control and other treatments. However, no statistical difference was apparent in NO levels in ASM cells after 24hr incubation. Nor was there any statistical difference in

NO expression in ASM cells after LPS/LXA4 treatment over 72hrs (**Fig.34**).

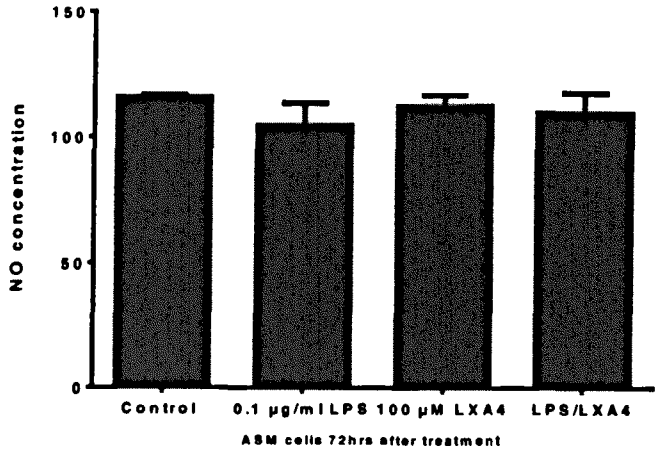

Figure 34: Quantative graph of fluorometric detection of NO expression in primary ASM cell supernatant after incubation with 0.1/ml LPS and/or 100µM LXA4 concentration over 72hrs. NO expression measured by fluorescent intensity (relative units to nitrite/nitrate standard curve)

Primary ASM cells were treated with 0.1µg/ml LPS, 100µM LXA4, or pre-treated for 15 minutes with 100µM LXA4 before LPS incubation. Primary equine ASM cells were obtained from trachealis muscle explants from the same tissue subject as from NO assay at 24hrs. Each error bar represents a group mean (SEM) obtained from 3 replicates means from a single experiment. A one-way ANOVA was performed to indicate any statistical significance

It was concluded that treatment of ASM cells with LPS or LXA4 had no effect on the expression of NO after 24 or 72hrs.

5.5 Discussion

The *in vitro* model of ASM cells successfully developed and characterised by the use of equine trachealis muscle explants was used for these experiments. The response of *in vitro* ASM cells to treatment with either LPS or LXA4 or both was examined to determine inflammation and inflammatory resolution.

One consideration to determining inflammation in respiratory smooth muscle was the potential response of *in vitro* ASM cells. The

characteristics of ASM cells, either *in vivo* or *in vitro* can demonstrate diverse biological reactions according to their environment. As such, *in vitro* ASM cells from trachealis muscle may exhibit synthetic, contractile and inflammatory phenotypes (Wright *et al* 2012). Smooth muscle cells *in vitro* are capable of converting between a contractile sessile state to a synthetic phenotype in response to stimuli such as mechanical strain and inflammation (Beamish *et al* 2010). ASM cells demonstrating a synthetic phenotype are able to respond to inflammation by proliferating, whereas the contractile type participates in bronchoconstriction (Wright *et al* 2012).

A potential disadvantage to culturing ASM cells with explants is that cells must undergo several doubling cycles before confluence (Hirst 2004). Such doubling is reported in vascular smooth muscle studies to reduce contractile properties in cells. Cultured equine ASM cells showed steady proliferation, and both baseline and inflammatory states were established. Accordingly, it was considered that a contractile phenotype from explant culture was not present and cells were suitable for investigations into inflammation.

ASM cells exhibit immune cell properties and in asthma demonstrate a pro-inflammatory phenotype (Damera & Panettieri 2011). Molecules known to stimulate ASM cells include expression of toll-like receptor TLR-4. TLR-4 mRNA and protein levels have been associated with ASM proliferation (Su *et al* 2012). TNF-α and IL-1β induce inflammation in human ASM cells (Keslacy *et al* 2007, Heffler *et al* 2007), and also act as potential mitogens for ASM (Hakonarson & Grunstein 2003, Tagaya & Tamaoki 2007, Nie *et al* 2011). Additionally, IL-1β and TNF-α mediate COX-2 expression in

human ASM cells (Belvisi *et al* 1997, Pang & Knox 1997). An *in vitro* ASM model was used to assess if equine ASM cells exhibited a similar inflammatory response to that observed in human studies. COX-2 mRNA was significantly increased after 72hrs incubation with 0.1 μg/ml LPS concentration (*p* value 0.0115), and this was considered to be a suitable time point and LPS concentration. An inflammatory response at 72hrs has also been observed in human airway smooth muscle cells at 72hrs (Xie *et al* 2005, Chen& Khalil 2006, Roth *et al* 2013). COX-2 mRNA has been detected in cultured human ASM cells at 12hrs with protein corresponding to COX-2 at 24hrs (Belvisi *et al* 1997). Peak expression of PGE2, an inflammatory molecule associated with COX-2 expression has been observed in human ASM cells at 24hrs (Pang & Knox 1997). In contrast, equine ASM cells did show noticeable levels of COX-2 at these times.

However, repeating incubation of ASM cells with 0.1 and 10μg/ml LPS again induced very similar mRNA levels of ALX, TLR-4, and TNF-α, IL-1β, iNOS and COX-2 at 72hrs. Previous experiments with ETE cells had used a reduced LPS concentration of 0.1μg/ml due to difficulties experienced with underlying inflammation in primary cells. This may have unduly influenced the decision to use the same LPS concentration for ASM cells. It should have been considered that ETE and ASM cells may not exhibit similar levels of immune defence. Bronchial epithelial cells are frequently cited as providing vital early defence for innate immunity. Although ASM cells have the ability to generate an inflammatory phenotype (Barnes 1998, Morris *et al* 2005, Damera & Panettieri 2011, Ramakrishna *et al*

2012), they are less likely to be as reactive as ETE cells, where respiratory defence is paramount in immunity.

Results for primary equine ASM cells at 72hrs with 0.1μg/ml LPS are in contrast to comparative studies of equine vascular smooth muscle cells *in vitro* where COX-2 mRNA expression was detected at 12, 24 and 48hrs after incubation of vascular smooth muscle cells with 10μg/ml LPS (Janicke *et al* 2003). Data to detect *in vitro* inflammation in ASM cells may have been more definitive if a time point of 48hrs had been included. The use of 72hrs in ASM studies of equine respiratory disease may have missed important inflammatory and inflammatory resolution points. Based on the similar results for ASM cells after 0.1 and 10μg/ml LPS concentrations and data obtained from equine vascular studies, it may have been preferable to examine the response of ASM cells to an increased LPS dose of 10μg/ml at earlier time points such as 48hrs.

Such alterations could have improved the data obtained for the LXA4 study. Comparative investigations into the effect of LXA4 on smooth muscle in human and horse are limited. LXA4 is thought to inhibit vascular smooth muscle cell chemotaxis thus regulating cellular remodelling (Parameswaran *et al* 2004). Unusually, although LXA4 activity was attributed to inhibition of the inflammatory molecule LTE4 no evidence of lipoxin receptor ALX expression was detected (Parameswaran *et al* 2007).

As with ETE cells, ASM cell samples showed very little difference between untreated cells and cells treated with LXA4 or LPS. A very marginal decrease of TNF-α and IL-1βmRNA at 24hrs after LXA4 incubation was

detected. This result was questionable, given that control and LPS samples produced very similar levels of expression. ALX mRNA appeared to be increased in the control group compared to the LPS treatment groups, but this was attributed to the noticeable variation in error bar values, as seen with ETE cells. Error bar anomalies were again thought to result from individual horse variation. This highlights the need for primary cells to be passaged and stocks of cells to be kept in reserve to ensure continuity in results.

Despite the inconsistent mRNA expression in LXA4 studies, some inhibition of COX-2 protein was observed after Western blot analysis. Bands corresponding to COX-2 protein were not detected at 72hrs in LXA4 and LPS/LXA4 treated samples. Absence of signal for previous Western blot results for COX-2 protein from ETE cells had been attributed to antibody inefficiency. Signal for COX-2 protein was more detectable in other ASM samples, and thus absence of COX-2 was accredited to LXA4 inhibition. Unlike results for previous LXA4 studies (Parameswaran *et al* 2007), bands corresponding to protein expression of ALX were strongly evident. It can be concluded that mRNA effects of LXA4 incubation may be extremely brief, but still able to partially inhibit formation of inflammatory proteins.

Similarly, detection of NO levels in ASM cells may also be hindered by brief expression of such molecules. An apparent minor difference observed in LXA4 treatment of ASM cells at 24hrs was lost at 72hrs, where no noticeable difference in treatment groups could be detected.

5.6 Conclusion

Use of *in vitro* ASM cells obtained from trachealis muscle explants were suitable for use in investigations of inflammation and inflammatory resolution. The initial detection of inflammation by significant expression of COX-2 mRNA in ASM cells at 72hrs after incubation with 0.1µg/ml LPS was not reproduced in subsequent experiments. Nor did LXA4 incubation apparently inhibit inflammation either at mRNA or protein levels. It was concluded that inflammation and inflammatory resolution is neither consistently induced nor noticeably resolved in primary equine ASM cells with the current chosen time points and incubation concentrations.

Chapter 6: An equine co-culture model of conditioned media from primary ETE cultures and ASM cells

6.1 Introduction

The properties of epithelial cells and their effect on other immune cells differ according to their inflammatory state (Levine 1995). Excessive inflammation from damaged respiratory epithelium causes bronchial smooth muscle alteration in airway disease (Al-Muhsen *et al* 2011). Attempts to develop more physiologically realistic *in vitro* respiratory models to investigate this effect include the co-culture of cells with conditioned media from other cell types. Conditioned media is considered able to support cell growth and activity (Xu *et al* 2005).

Data detailing conditioned media in equine co-culture studies from airway disease in horses is limited. Currently comparable works of equine co-culture studies are restricted to studies of inflammatory changes in equine cartilage in response to endogenous cell activity (MacDonald & Benton 1996, Gregg *at al* 2006). *In vitro* co-culture methods offer the opportunity to study cellular interactions in the pathogenesis of inflammatory diseases such as RAO on a more advanced level.

6.2 Aims and objectives

- To characterise the response of primary ASM cells to incubation with conditioned media from primary ETE cells
- To determine the potential for conditioned media from ETE cells to induce inflammation or inflammatory resolution in ASM cells by assessment of selected molecules

To achieve these aims, primary *in vitro* ETE and ASM cells were obtained and cultured as described (Chapter 3). ETE cell samples were treated with 0.1µg/ml LPS and 100µM LX4 concentrations for 24hrs prior to harvesting supernatant medium. The response of ASM cells to treatment with supernatant from ETE cells was analysed by quantification of mRNA and detection of protein expression.

6.3 Methods

Primary ETE cells were collected by use of the reduced trypsin incubation method. Equine ASM cells were again obtained from the out-growth of cells from trachealis muscle explants. Primary ETE and ASM cells were maintained in BEGM or serum-supplemented DMEM media respectively until 70-75% confluent before use in experiments. Primary ETE and ASM cells were not passaged prior to co-culture studies. Control samples for co-culture investigations did not receive either LXA4 or LPS treatment.

ETE cells were then incubated for 24 hours with 0.1µg/ml LPS, 100µM LXA4 or pre-treated with 100µM LXA4 for 15mins before 24hr incubation with 0.1µg/ml LPS. Media from treated ETE cells was collected, sterile

filtered and incubated with cultured ASM cells for a further 24hrs. Control samples for ETE cells did not receive either LXA4 or LPS treatment. The response of ASM cells to co-culture with conditioned media from ETE cells was assessed by the same methods as Chapter 3, and 4 with full details in Chapter 2.

6.4 Results

ASM mRNA expression after co-culture with conditioned media from ETE cells treated with 0.1μg/ml LPS, 100μM LXA4 and/or LPS/LXA4 concentrations

To determine if an inflammatory response could be transferred from ETE cells to ASM cells, a co-culture with conditioned media was investigated. ETE cells were treated with the concentrations stated in methods for 24hrs, and this media used in cultures of ASM cells for a further 24hrs.

Use of conditioned media from treated ETE cells did not noticeably stimulate a response in ASM cells after 24hrs incubation. Expression of mRNA in control and LXA4 treated samples from co-cultured ASM cells for ALX, TLR-4, TNF-α, IL-1β and iNOS was not apparent. This lack of obvious mRNA levels indicative of inflammation in control and LXA4 samples suggested ASM cells were in a non-inflammatory state. Levels of mRNA were more apparent in LPS and LPS/LXA4 treated samples, potentially indicating the inflammatory capacity of ETE cell conditioned media on ASM cells (**Fig.35**).

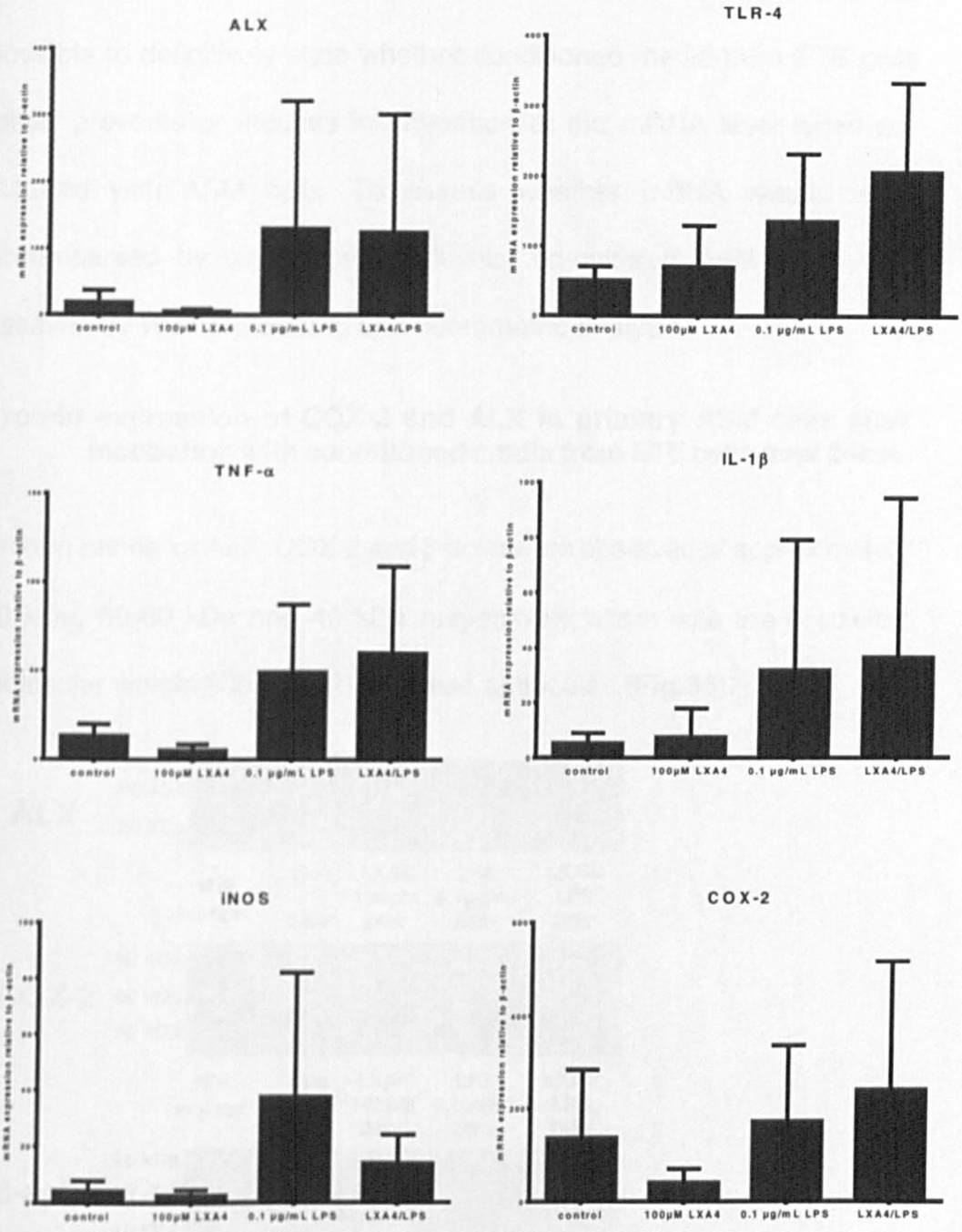

Figure 35: q-PCR graph of ALX, TLR-4, TNF-α, IL-1β, iNOS and COX-2 mRNA expression in ASM cells after 24hr incubation with conditioned media from ETE cells

ETE cells (obtained from reduced trypsin digestion method) received 24hrs treatment with 0.1μg/ml LPS, 100μM LXA4 and/or LPS/LXA4. Error bars represents a group mean (SEM) obtained from 3 sample means from three separate tissue subjects to previous experiments. A one-way ANOVA was performed to indicate any statistical significance.

Substantial disparity in SEM error bars was observed in all mRNA expression for LPS and LXA4/LPS samples. Statistical analysis of results by one-way ANOVA did not produce any significant results. It was

concluded that due to the considerable variation in results, it was not possible to definitively state whether conditioned media from ETE cells either prevents or induces inflammation at the mRNA level when co-cultured with ASM cells. To assess whether mRNA results were accompanied by similar protein levels, co-cultured ASM cells were assayed by Western blotting and fluorometric analysis.

Protein expression of COX-2 and ALX in primary ASM cells after incubation with conditioned media from ETE cells over 24hrs.

Protein bands for ALX, COX-2 and β-actin were observed at approximately 30 kDa, 60-80 kDa and 40 kDa respectively which was the predicted molecular weight (Chapter 2) for these antibodies (**Fig.36**).

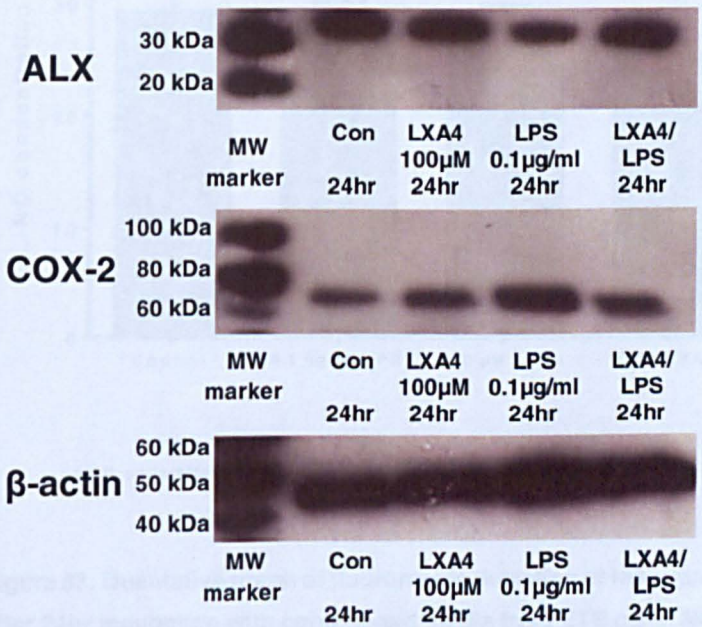

Figure 36: Western blot image of protein expression of β-actin, COX-2 and ALX in primary ASM cells after incubation with conditioned media from ETE cells over 24hrs

ASM cells were incubated for 24hrs with conditioned media from ETE cells obtained from reduced trypsin digestion method. ETE cells received 24hrs treatment with 0.1μg/ml LPS, 100μM LXA4 and/or LPS/LXA4. Media from treated ETE cells were incubated with ASM cells for a further 24hrs. Primary cells were obtained from a separate tissue subject to previous experiments.

No significant difference was apparent in protein expression in all ASM cell samples. It was concluded that, as with mRNA expression, conditioned media from ETE cells did not produce an observable change in COX-2/ALX protein expression in ASM cells after 24hrs.

Production of NO in primary ASM cells after incubation with conditioned media from ETE cells over 24hrs

To determine if conditioned media from ETE cells increased/decreased NO expression in ASM cells, supernatant from ASM co-cultured cells was assayed for NO expression by fluorometric analysis (**Fig.37**).

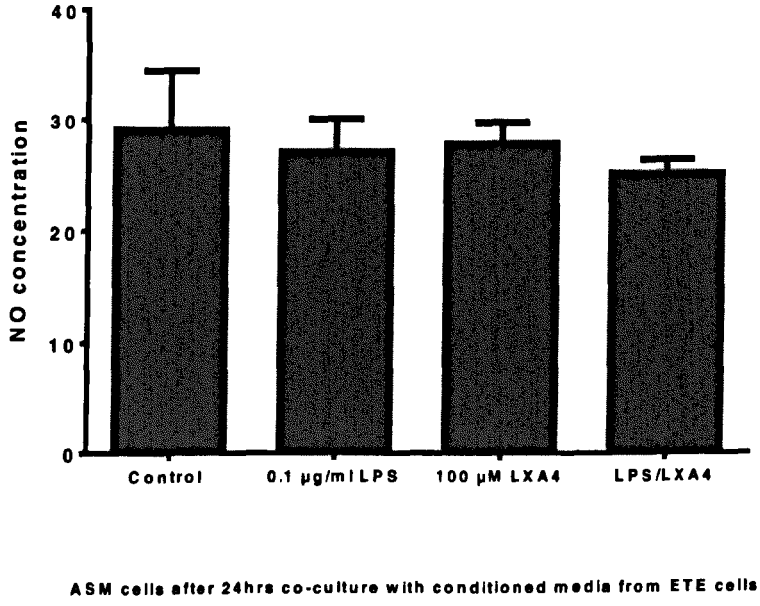

Figure 37: Quantative graph of fluorometric detection of NO expression in primary ASM cells after 24hr incubation with conditioned media from ETE cells. NO expression measured by fluorescent intensity (relative units to nitrite/nitrate standard curve)

ETE cells received 24hrs treatment with 0.1µg/ml LPS, 100µM LXA4 and/or LPS/LXA4. Treated ETE media was then used to incubate ASM cells for a further 24hrs. ASM cells were incubated for 24hrs with conditioned media from ETE cells obtained from reduced trypsin digestion method. Primary cells were obtained from a separate tissue subject to previous experiments. A one-way ANOVA was performed to indicate any statistical significance.

In a similar manner to the results observed for mRNA and protein levels, there was no distinct difference in treatment groups for ASM cells incubated with conditioned media from ETE cells. It was concluded that co-culture of ASM cells by use of conditioned media from ETE cells neither induces nor inhibits NO expression in ASM cells.

6.5 Discussion

Co-culture with conditioned media offers a simple method to study *in vitro* inflammation between the epithelial and the bronchial smooth muscle layer. The capacity to distinguish mediators of inflammation in epithelial and airway smooth muscle cells contributes to increased understanding of RAO pathogenesis.

Use of conditioned media has worked well for human studies of bronchial epithelial cells of airway inflammation and ASM remodelling (Tang *et al* 2012, Kuo *et al* 2011, Takeda *et al* 2009). Conditioned media from resting BEAS-2B cells and primary murine tracheal epithelial cells has inhibited TNF-α expression in monocytes, macrophages and dendritic cells (Mayer *et al* 2008). Airway alterations have been modelled by a human bronchial epithelial cell line 16HBE14o. Conditioned media obtained from mechanically damaged 16HBE14o cells induced significant proliferation of myofibroblasts (Zhang *et al* 1999). Media from transformed human bronchial epithelial cells (BEAS-2B) cells was capable of relaxing pre-contracted adult pulmonary arterial muscle (Farah *et al* 2009).

In the current study, investigations in to the relationship between bronchial epithelium and smooth muscle layer were unsuccessful. Conditioned

media from treated primary ETE cells used to stimulate primary ASM cells did not elicit a noticeably different response in treated and untreated samples. Nor was there any similarity shown in the response of ASM cells incubated with conditioned media from ETE cells to that of ASM cells which were treated similarly with LPS and/or LXA4 in Chapter 5. Results obtained with q-PCR using LXA4 treated ETE and ASM cells were very similar to those of ASM cells cultured in conditioned media from LXA4 treated ETE cells. Data was again ambiguous due to the highly variation shown in the SEM bars. The lack of significant response from ASM cells may be due the absence of molecules in conditioned ETE cell media able to promote or resolve inflammation.

Previously, 0.1 and 10μg/ml of LPS concentrations produced closely similar mRNA expression in ASM cells, with results obtained after 72hrs incubation. Thus it seems unlikely that a shorter incubation period of 24hrs with unknown levels of potentially inflammatory products would elicit a response. The use of ELISA assays on collected ETE cell media could have been used to determine the presence of inflammatory molecules such as TNF-α or IL-1β prior to use for co-culture.

ASM cells may produce an inflammatory reaction to either early acute inflammation or from repeated episodes of inflammatory insult. One alternative would have been to collect increased quantities of conditioned media from the same ETE cell samples. Collected media could then have been used to change media for ASM cells every 24hrs over a 72hr period. This would therefore replicate repeated inflammation *in vitro* and extend the incubation time to the 72hrs time point used for ASM cells.

One hindrance to obtaining results with conditioned media is the change in culture media for ASM cells. Primary ETE cells require BEGM media, formulated to be serum free. Equine ASM cells were dependant on the presence of BSA during culture to promote cell growth. Serum withdrawal has been implicated as a factor in reduction of ASM cell activity. Serum depletion is frequently used in studies of cell cycles and their metabolism (Pirkmajer & Chibalin 2011). However serum withdrawal from cell media is linked to alterations in cell signalling and gene expression (Zander & Bemark 2011). Therefore use of BEGM serum-free media from ETE cells could have affected data obtained from ASM cells in co-culture studies. The addition of serum to ETE cell conditioned BEGM media was thought likely to affect potential results.

One alternative could have explored the effects of a reduction of serum levels in ASM culture media. Assessment of the suitability of cell culture media can be achieved by measuring cell carbohydrate activity. Quantifying the molar concentration of lactate in cell media indicates the level of glucose uptake and subsequently, the hypoxic qualities of cell media used (Gstraunthaler *et al* 1999). This could have improved monitoring of ASM activity and reaction to serum reduction or change to BEGM media. It would have also aided the selection of a suitable media for both ETE and ASM cells. Finding an appropriate media for both cell types would enhance the development of an ALI co-culture system between ETE and ASM cells. Initial efforts were made to culture primary ETE cells with tissue culture inserts at the ALI (data not shown). Primary ETE ells were cultured at the ALI with BEGM, DMEM and with L-15, but

did not appear to proliferate when viewed under the light microscope. Development of an *in vitro* ALI co-culture model between ETE and ASM cells is a key aim for future work.

Western blotting did not appear to show any significant difference in treatment groups for COX-2 or ALX. There appeared to be a very minor difference in the strength of signal for COX-2 compared with ALX. COX-2 seemed to be more strongly expressed compared with ALX after LPS incubation of samples. To enable a more accurate assessment of protein levels, semi-quantative analysis of Western blotting should have been performed on results.

Samples used in NO analysis were again unconvincing and showed no difference in sample levels. As described in the discussion for chapter 3, NO expression is often affected by several variables in cell culture with NO expression frequently limited. The same variables were thought to affect results for co-cultured ASM cells.

6.6 Conclusion

In conclusion, it appears that conditioned media from ETE cells appears to have no significant effect on NO expression in ASM cells after 24hrs. At present, the use of conditioned media as a model of the interactions between these two cell types is unsuitable for investigations in to RAO It may be that after adjustment, this method could be used to investigate the relationship between ETE and ASM cells.

Chapter 7: General Discussion and Conclusions

An *in vitro* equine respiratory model was successfully developed by culture of primary ETE and ASM cells. Use of an equine *in vitro* cell culture model represents a feasible method of studying bronchial inflammation and inflammatory resolution contributing to RAO pathogenesis.

The horse is an excellent animal model to study human airway disorders. No animal model is completely representative of human asthma, but horses with RAO show naturally occurring clinical symptoms similar to asthma after exposure to organic dust. Furthermore, as a large animal, the horse offers a greater physiological similarity to people than small animal models of respiratory disease (Zosky & Sly 2007).

Development of the model required extensive assessment of multiple *in vitro* methods, which delayed investigations into inflammation and inflammatory resolution. Use of the continuous cell line BEAS-2B would have significantly increased the rate of investigation into airway inflammation. Despite the reliability and practicality of an immortalized cell line, BEAS-2B cells were not used as they were considered to lack phenotypic and differential characteristics of the original airway tissue.

Developing an *in vitro* smooth muscle model of primary equine ASM cells from culture of trachealis muscle explants proved uncomplicated. Cultured ASM cells showed a morphology and growth pattern highly similar to other equine studies of primary smooth muscle (Rodgerson *et al* 2002, Janicke *et al* 2003). Characterisation of ASM cells showed positive staining by

IHC/ICC labelling of α-smooth muscle actin and results were comparable with other studies culturing smooth muscle cells.

In contrast, establishing an *in vitro* epithelial model required extensive optimisation. Compared with similar works (Lin *et al* 2001, Vandekerckhove *et al* 2009), whole organ cultures proved unsatisfactory due to poor tissue viability or were unsuitable for investigations into inflammatory resolution.

Additionally, unlike several primary bronchial epithelial cell culture studies (Spurzem *at al* 2005, Kato *et al* 2006, Newby *et al* 2007, Erles & Browlie 2010, Newby *et al* 2007), digestion of equine respiratory tissue with pronase to obtain cells was unproductive. Isolation of bronchial epithelial cells from pronase digestion of respiratory tissue has been very successful for other equine studies (Lin *et al* 2002, Ainsworth *et al* 2009, Quintana *et al* 2011). Work in these studies is particularly relevant, as the authors subsequently investigated the effects of LPS on primary equine bronchial epithelial cells (Ainsworth *et al* 2009), and the development of an immunological profile (Quintana *et al* 2011). Therefore not following the primary culture methods described in these works more fully may have been an important error. It would have been preferable to have attempted the described protocols more extensively, particularly by use of increased digestion times (up to 48hrs) and the addition of supplemented digestion media (e.g. DNase) described in these studies.

Despite this, use of trypsin digested tissue produced adequate quantities of proliferating ETE cells, although average numbers and viability of cells

obtained did not equal those described in the original paper (Shibeshi *et al* 2008). ETE cells were characterised by similar IHC/ICC positive staining of cytokeratin protein for epithelial cells with PCK-26 as also described in the original paper. Use of trypsin-dissociated ETE cells for the investigation of RAO was novel and original. Additionally the culture and characterisation of airway smooth muscle cells in the horse has not been detailed elsewhere. Nor has an *in vitro* model composed of ETE and ASM cells been previously described in the literature.

However, further development of both ETE and ASM cells is required in both characterisation and production of cell banks. For future work, it would be preferable to improve characterisation of primary ASM cells to fully distinguish ASM cells from myofibroblasts. This could be achieved by use of additional markers to α-smooth muscle actin to include other smooth muscle proteins, such as smooth muscle myosin heavy chain (sm-MHC), calponin, and desmin (Halayko *et al* 1996).

Semi-immortalization of primary cells would be one potential alternative, particularly given the lack of equine continuous cell lines currently available. Semi-immortalization of primary epithelial cells has been achieved through transfection of primate tissue explants with Epstein Barr Virus DNA (Gao *et al* 2002). A more practical immediate option would be establishing a successful method to passage both ETE and ASM cells. Further development of trypsin dissociated cells has been described by Abraham *et al* (2011). Primary ETE cells obtained in this manner were successfully passaged and were able to fully differentiate into pseudo-stratified muco-ciliary epithelium with basal cells when cultured at the AFI.

Passaging of ETE cells may have been particularly important as significant COX-2 mRNA expression ($p< 0.0001$) was observed in freshly dissociated cells after 5 days of culture. It was not anticipated that passaging of ETE cells would eliminate trypsin-associated inflammation. It is still an important error during model development as it had been fully detailed in the original methodology and should have been attempted.

Creating reserves of primary ETE and ASM cells would enhance continuity between experiments and the validity of data obtained from such investigations. Reducing the number of tissue donors to obtain primary cells would prevent variety in obtaining data. Noticeable variability in data was observed during investigations into inflammation in ETE and ASM cells. Initially, persistent inflammation unrelated to LPS stimulation was evident in ETE cells obtained after 2hrs trypsin dissociation of tracheal tissue. Incubation with anti-inflammatory carprofen did not eliminate this inflammation, in a similar manner to other studies reporting the effectiveness of *in vitro* carprofen on equine cells (Brideau *et al* 2001, Beretta *et al* 2005). An apparent decrease of COX-2 mRNA associated inflammation in ETE cells was subsequently established by use of reduced incubation with trypsin during cell dissociation. It was considered that the reduced trypsin digestion method established baseline levels of mRNA expression in ETE cells. No evidence of obvious prior inflammation was observed in ASM cells obtained from trachealis explants.

The response of ETE and ASM cells was examined after stimulation with LPS concentrations. The use of 0.1 µg/ml LPS concentration to induce inflammation in ETE and ASM at 24 and 72hrs respectively was partially

successful compared with control samples and other LPS concentrations. Significant increases were observed in ETE mRNA for ALX, TLR-4, TNF-α and IL-1β, and initial COX-2 mRNA investigations into inflammation in ASM cells (all p values < 0.05). No statistical significance was evident for ETE cells expression of mRNA for iNOS and COX-2. Extensive optimisation of the methods used to detect inflammation in ETE and ASM cells is required.

However, definitively establishing basal expression of selected molecules in ETE and ASM cells may be challenging. Primary human tracheal epithelial cell cultures grown at the air-liquid interface have not demonstrated transcriptional differences to those of *in vivo* airway epithelia (Pezzulo *et al* 2010). In contrast, an immunological profile was established (Quintana *et al* 2011), with primary equine epithelial cells cultured at the ALI. Despite no stimulation of fully differentiated cells with inflammatory molecules, expression of IL-8, TNF-α and IL-6 were detected in the model after 4 weeks. The presence of these molecules indicates low level or persistent inflammation which would affect studies of inflammation or inflammatory resolution. Nor has an immunological profile for *in vitro* equine ASM been determined. Studies into human ASM indicate that *in vitro* ASM has an inflammatory phenotype, which equally hinders studies of inflammation (Damera & Panettieri 2011).

Statistical analysis of collected data was performed with both one and two way ANOVA and corroborated by Tukey's multiple comparison tests. Use of these tests was determined by the distribution pattern of collected data. Parametric tests can place rigid restrictions on results with assumptions of

normally distributed Gaussian numbers. However, it was considered to be the most appropriate test, which would then highlight any statistically significant data. To improve the reliability and validity of collected data, alterations to the number of genes used and the selected time points are necessary. Reduction in the number of genes examined is desirable to aid repeatability of experiments. Examination of genes more closely associated with the early initiation of inflammation such as TNF-α or IL-1β, rather than COX-2 should improve detection of acute inflammatory onset. Additionally, as previously mentioned, the selected time points of 24 and 72hrs may be unsuitable for quantification of mRNA expression.

A final and important factor in reducing variability in data was the source of tissue used in experiments. Data for inflammation studies which examined COX-2 mRNA expression were obtained from primary cells collected from a separate horse for each experiment. Similarly, LXA4 data was resulted from tissue obtained from three separate horses. Both factors induce considerable scope for variation in cell expression due to the potential differences in each subject's health and history.

Consideration should also be given to the use of tracheal tissue from the upper respiratory tract to examine RAO. Human airway epithelial cells from trachea and bronchi have high levels of similarity and expression profiles (Pezzulo *et al* 2010). Additionally, equine mucosal explants collected from septum, the nasopharynx and the trachea and maintained at the AFI showed no difference in viability determined by TUNEL staining (Vandekerckhove *et al* 2011).

However, RAO is a disorder affecting the lower respiratory tract and comparable studies which have successfully instigated inflammation after LPS stimulation have used lung bronchi (Ainsworth *et al* 2009, Lin *et al* 2001). Therefore the use of tracheal tissue to investigate inflammation of the lower respiratory tract may not have been suitable.

Although an *in vitro* model of primary ETE and ASM cells was successfully established, it is clear that induction of inflammation in the model is not reliable. Use of peripheral blood monocytes cells (PBMCs) isolated from whole blood as described in Chapter 2 offers an alternative to the model with several advantages. Samples could be obtained from a reduced number of donors (complete with a known history, definitive ages and health score), therefore restricting the variability evident in results from different tissue sources. Use of whole blood samples also reduces the potential contamination from tissue collection and processing, and effect of tissue viability after transport. Increased access to regular numbers of cells also improves the opportunity to examine increased numbers of earlier time points.

An important factor in use of peripheral blood monocytes is the potential improved expression of receptor ALX. This is the first time that the effect of exogenous LXA4 on acute inflammation in primary ETE and ASM cells has been investigated. Detecting irregularities in the synthesis of LXA4 and its receptor ALX in horses offers a valuable diagnostic tool to investigate RAO.

Expression of the lipoxin receptor ALX has not previously been detected in primary ETE or ASM cells. It was therefore an unknown variable and

determining inflammatory resolution solely by expression of ALX could have been a limiting factor. In order to obtain q-PCR standards of sufficient quality for ALX, it was necessary to use a pooled selection of mRNA from ETE, ASM and PBMCs. Although ALX is known to be expressed in human cells, it is also expressed in monocytes and lymphocytes (Serhan 2005). Such cells are present in cell populations obtained from PBMCs and may show increased levels of ALX mRNA compared with epithelial cells. Establishing definitive levels inflammation and inflammatory resolution in PBMCs would aid similar detection in ETE and ASM cells.

There is also potential for the ALX receptor to be poorly expressed in equine ETE cells, and this could be an interesting point to establish for future RAO investigations. The LXA4 receptor ALX is a surface G-protein coupled receptor whose biological functions are not fully understood. ALX has an affinity for LXA4 but is able to bind to other substrates which could potentially reduce LXA4 action (Chiang 2002). ALX is capable of binding bacterial peptide tripeptide formyl-Methionine–Leucine–Phenylalanine (fMLF) and its analogues derived from *Escherichia coli* (Le *et al* 2001). The ALX receptor in BEAS-2B cells increased in response to incubation with LPS, TNF-α and to scratch injury (Shao *et al* 2011). ALX is also reported to bind to acute phase protein serum amyloid A (SAA), a protein associated with lung inflammation in people with COPD (Bozinovski *et al* 2012). Variation in the capacity of ALX to bind to available substrates would explain in part the results observed for treatment of ETE and ASM cells with LXA4/LPS concentrations

It is very likely that the LXA4 concentration used did not sufficiently stimulate ETE or ASM cells to activate the ALX receptor. This was in contrast a similar study where treatment of human bronchial epithelial cells after acid injury with 100μM of LXA4 was associated with expression of ALX and decreased IL-6 mRNA expression (Bonnans *et al* 2006). Variation in ALX expression was observed in an *in vitro* model of equine tendon explants (Dakin *et al* 2012). Expression of LXA4 (approximately 100 pg/ml) was observed at 24hrs after IL-1β stimulation with peak levels of ALX recorded at 72hrs.

However, the immune responses of different cell types (tenocytes compared with ETE/ASM cells) may be dissimilar and as such LXA4/ALX binding will also differ. To determine such binding a time and concentration course of exogenous LXA4 on ALX expression should be performed in a similar manner to selection of a suitable LPS concentration.

Conclusion

Culture of primary ETE and ASM cells are suitable for use as an *in vitro* respiratory model. Treatment of the model with LPS induces detectable inflammation in cultured ETE and ASM cells. However, such inflammation is not sufficiently repeatable nor is there conclusive or definitive evidence of inflammatory inhibition mediated by exogenous LXA4. It must be concluded that the obtained data is not able to support the hypothesis which must therefore be rejected.

As such, further optimisation of the model is required by:

- Continued characterisation of ETE and ASM cells or use of alternative cells types
- alterations to chosen time points of detection
- reduction in number of markers of inflammation used

Modifications to the existing model may then enable detection of potential inflammatory resolution associated with RAO and support the original hypothesis.

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Appendices

Appendix 1: Untreated data

Expression of mRNA for COX-2 in ETEC after 0, 4 and 24 hrs incubation with 10µg/mL LPS in ETEC (1 sample)

Treatment groups	COX-2/GAPDH ratio mean
Control 0hr	0.3027
Control 4hr	0.4967
Control 24hr	2.0875
LPS 0hr	0.3514
LPS 4hr	1.1606
LPS 24hr	1.7096

Expression of mRNA for COX-2 in ETEC after 0, 4 and 24 hrs incubation with 10µg/mL LPS and 4µg/ml carprofen

Treatment groups	COX-2/18S ratio mean
Control 0hr	0.05702
Control 4hr	0.26787
Control 24hr	0.62965
Carprofen 0hr	0.21653
Carprofen 4hr	0.14274
Carprofen 24hr	0.33566
LPS 0hr	0.10459
LPS 4hr	0.20525
LPS 24hr	0.46303
Carprofen/LPS 0hr	0.07474
Carprofen/LPS 4hr	0.29406
Carprofen/LPS 24hr	0.21082

Expression of mRNA for COX-2 expression in 5 day and 4 week old ETEC after incubation

Treatment groups	COX-2/Beta actin ratio mean
Control 0hr 5 day	0.0017
Control 24hr 5 day	0.11247
Control 0hr 4 week	0.00120
Control 24hr 4 week	0.02293

Expression of mRNA in ETEC after 0.1, 10 and 100 µg/ml LPS over 24 hrs (1 sample)

Treatment groups	Target/HK ratio mean	Time zero ratio	Normalised target gene
Control	**Control**	**Control**	**Control**
ALX	0.0014	0.0095	0.1551
TLR-4	0.0558	0.0501	1.1143
TNF-α	0.0160	0.2026	0.0792
IL-1β	0.0004	0.0006	0.7748
iNOS	0.0458	0.0488	0.9389
COX-2	0.1091	0.1204	0.9063
0.1 µg/mL	**0.1 µg/mL**	**0.1 µg/mL**	**0.1 µg/mL**
ALX	0.0354	0.0095	3.7079
TLR-4	0.1444	0.0501	2.8803
TNF-α	0.0852	0.2026	0.4208
IL-1β	0.0043	0.0006	7.1519
iNOS	0.0960	0.0488	1.9670
COX-2	0.8818	0.1204	7.3225
10 µg/mL	**10 µg/mL**	**10 µg/mL**	
ALX	0.0020	0.0095	0.2151
TLR-4	0.0841	0.0501	1.6787
TNF-α	0.0115	0.2026	0.0570
IL-1β	0.0009	0.0006	1.5306
iNOS	0.0545	0.0488	1.1180
COX-2	0.4284	0.12043	3.5576
100 µg/mL LPS	**100 µg/mL LPS**	**100 µg/mL LPS**	**100 µg/mL LPS**
ALX	0.0046	0.0095	0.4908
TLR-4	0.0516	0.0501	1.0291
TNF-α	0.0309	0.2026	0.1525
IL-1β	0.0008	0.0006	1.3732
iNOS	0.0430	0.0488	0.8815
COX-2	0.1091	0.1204	0.9065

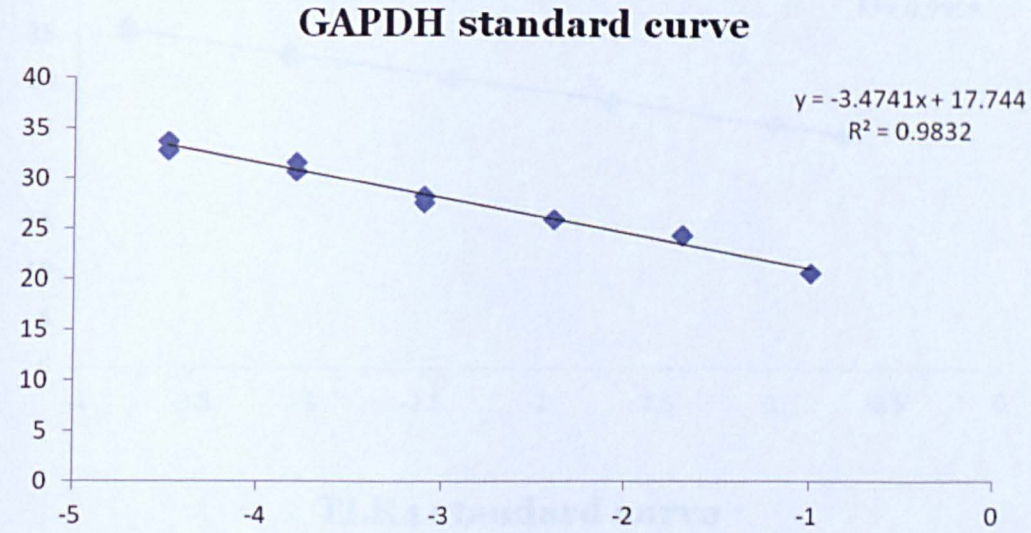
Expression of mRNA in ETEC after LXA4 and LPS incubation over 24hrs (3 samples)

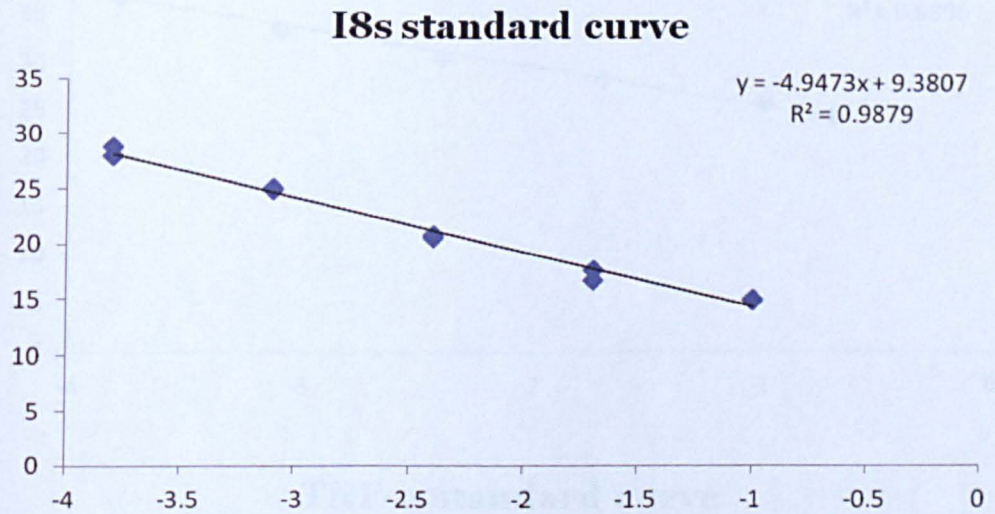
Treatment groups		
ALX	**Time zero ratio**	**Average of normalised samples**
Control	0.0022	1.1333
100μM LXA4	0.0022	1.7432
0.1μG/ml LPS	0.0022	1.4416
LPS/LXA4	0.0022	6.0002
TLR-4		
Control	0.01025	3.9682
100μM LXA4	0.01025	8.0945
0.1μG/ml LPS	0.01025	5.6793
LPS/LXA4	0.01025	5.2443
TNF-α		
Control	0.00439	77.1092
100μM LXA4	0.00439	36.1645
0.1μG/ml LPS	0.00439	86.9177
LPS/LXA4	0.00439	309.7666
IL-1β		
Control	0.0001	5.5475
100μM LXA4	0.0001	4.6752
0.1μG/ml LPS	0.0001	9.8018
LPS/LXA4	0.0001	20.1245
iNOS		
Control	0.0012	24.4892
100μM LXA4	0.0012	91.5493
0.1μG/ml LPS	0.0012	66.6176
LPS/LXA4	0.0012	30.9618
COX-2		
Control	0.0011	22.7333
100μM LXA4	0.0011	111.9172
0.1μG/ml LPS	0.0011	91.6903
LPS/LXA4	0.0011	127.2503

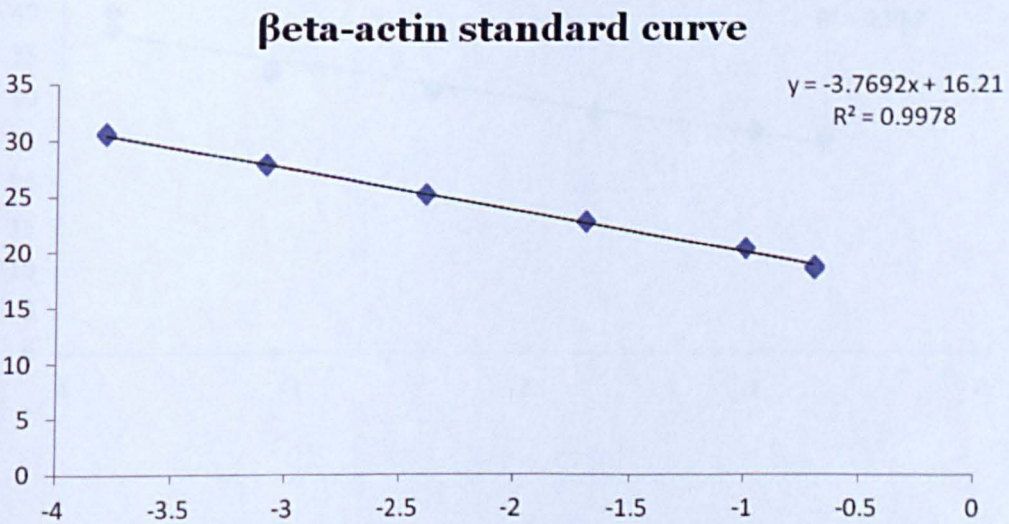
Co- culture studies

ALX	Time zero ratio	normalised mean samples
control	0.0003	0.0034
LXA4	0.0003	0.0007
LPS	0.0003	0.0062
LXA4/LPS	0.0003	0.0025
TLR-4		
control	0.0009	0.01355
LXA4	0.0009	0.02220
LPS	0.0009	0.02517
LXA4/LPS	0.0009	0.03077
TNF-A		
control	0.0014	0.01574
LXA4	0.0014	0.00617
LPS	0.0014	0.02717
LXA4/LPS	0.0014	0.03151
IL-1B		
control	0.0002	0.0004
LXA4	0.0002	0.0002
LPS	0.0002	0.0006
LXA4/LPS	0.0002	0.0005
iNOS		
control	0.0031	0.0038
LXA4	0.0031	0.0067
LPS	0.0031	0.0091
LXA4/LPS	0.0031	0.0053
COX-2		
control	0.0119	0.0216
LXA4	0.0119	0.0564
LPS	0.0119	0.0926
LXA4/LPS	0.0119	0.0802

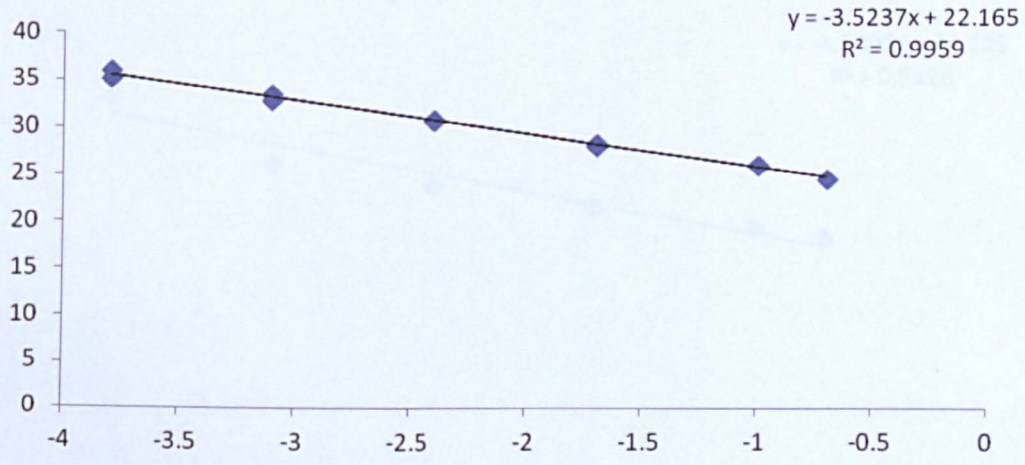
Appendix 2: PCR standard curves, melt curves and reference genes



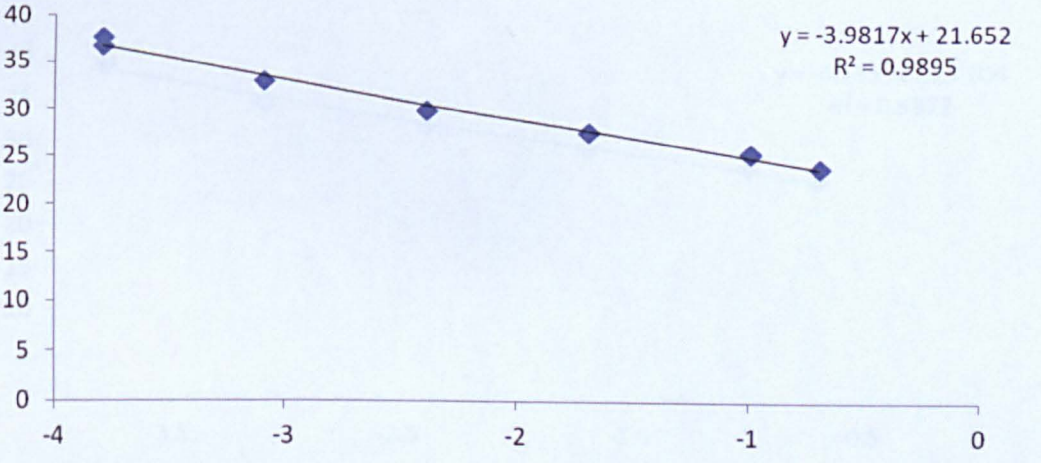




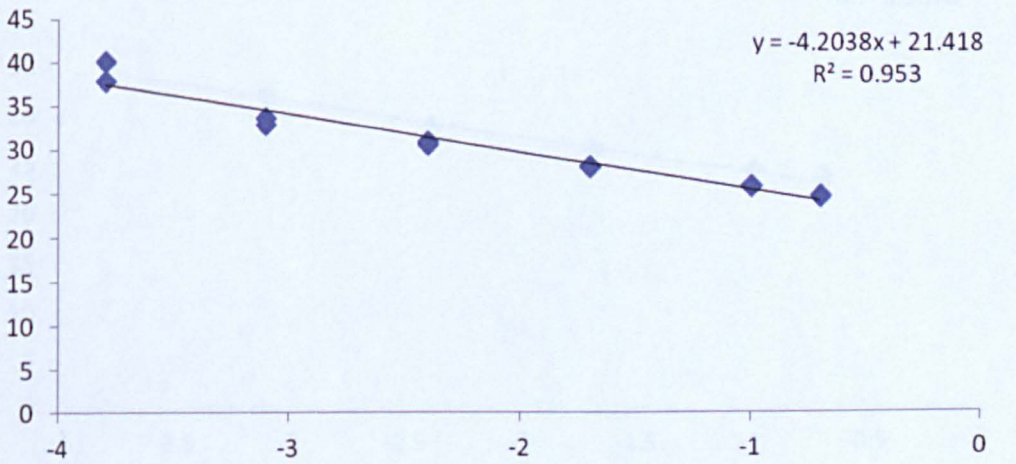
ALX standard curve



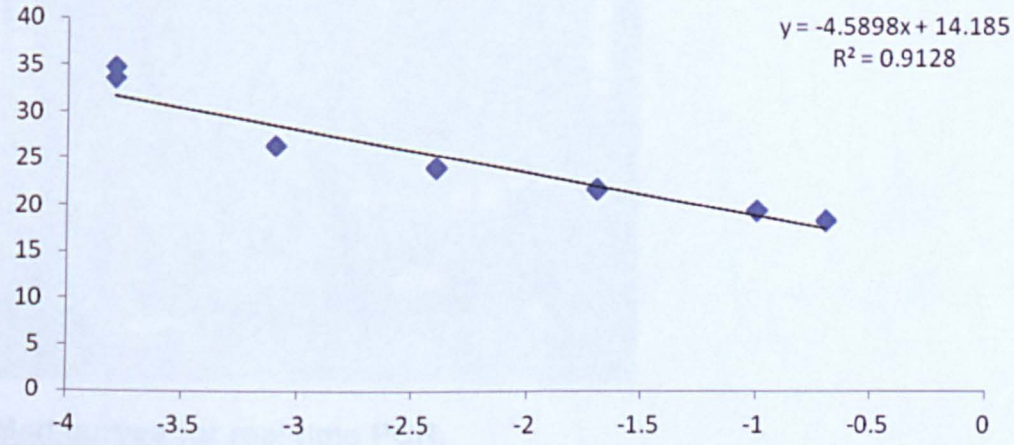
TLR4 standard curve



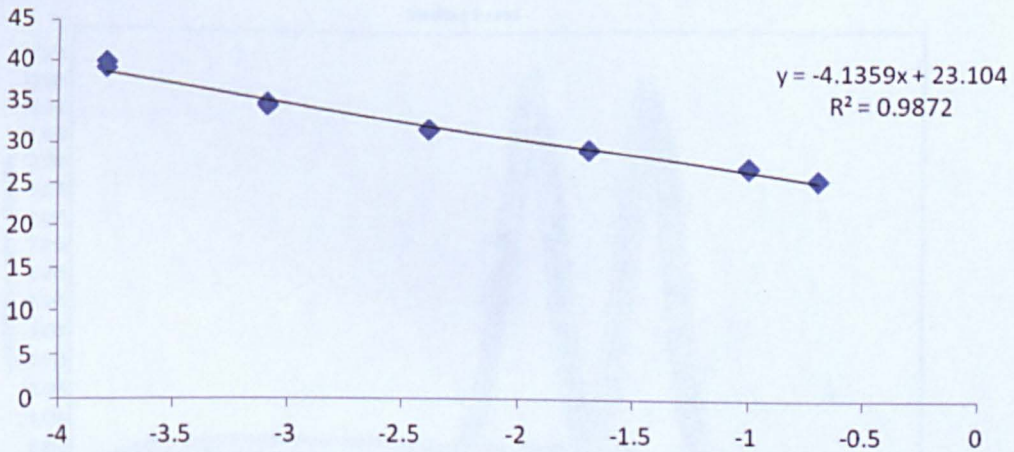
TNF-α standard curve



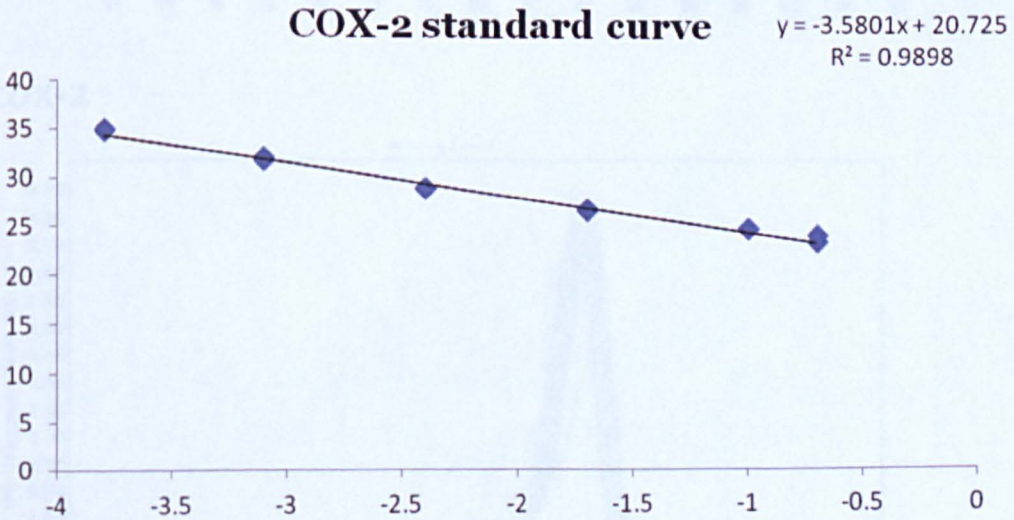
IL-1β standard curve



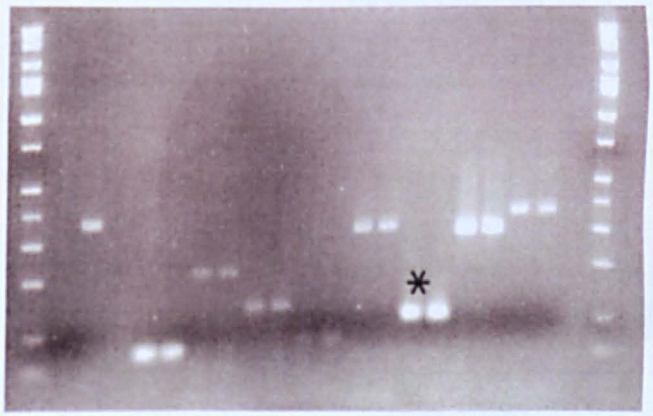
iNOS standard curve



COX-2 standard curve

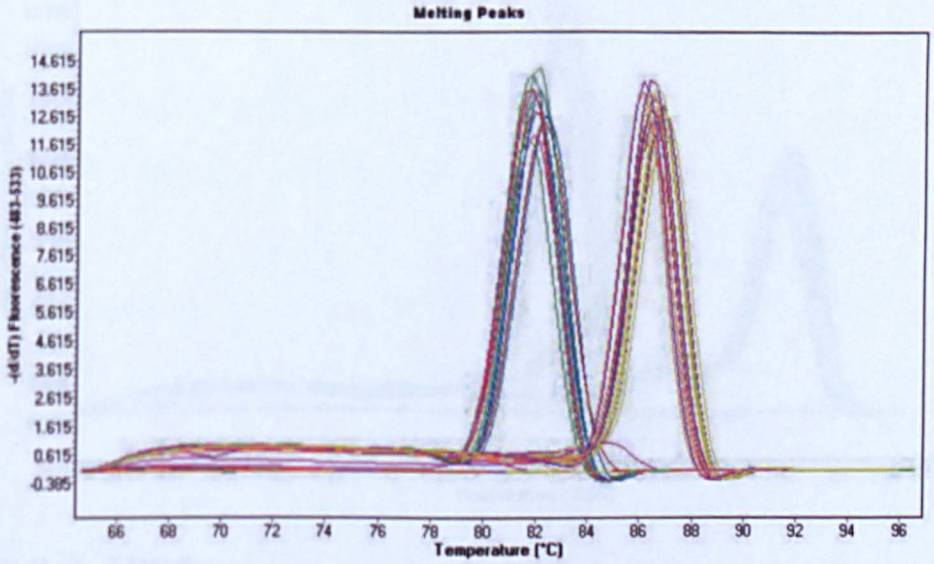


End-point PCR of reference gene Beta-actin (*)

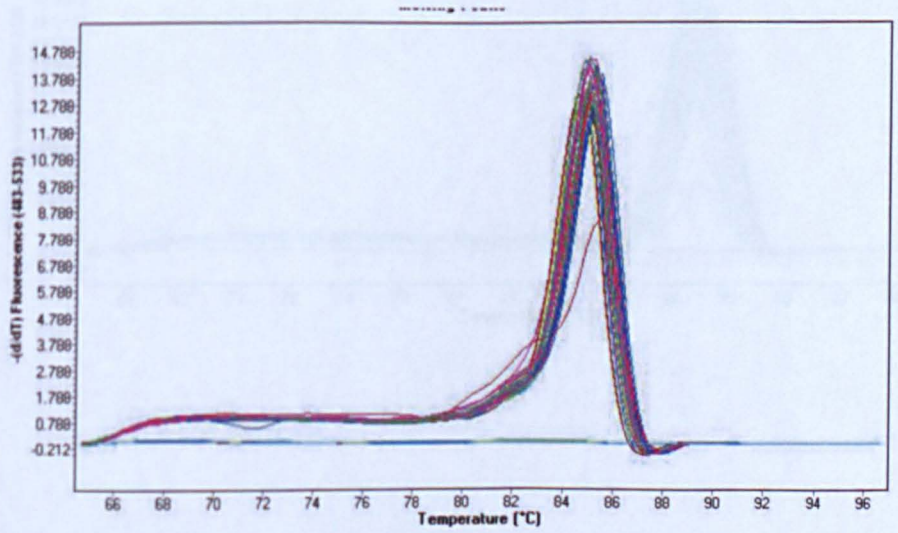


Melt curves for real time PCR.

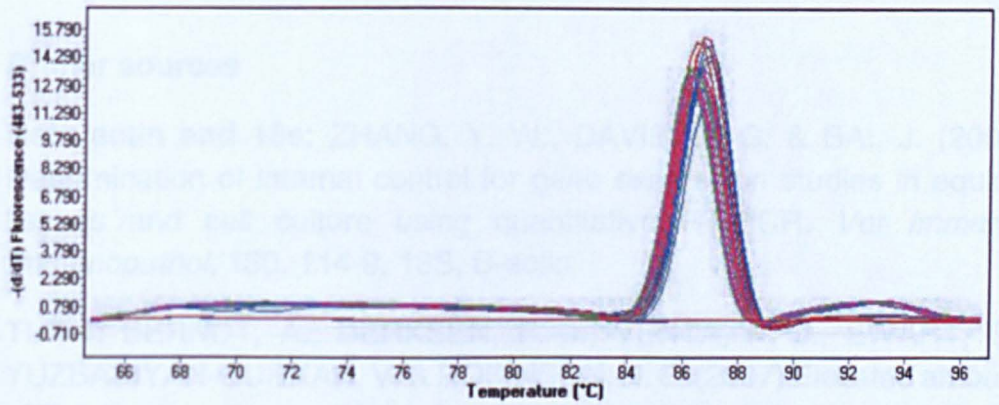
ALX and 18S



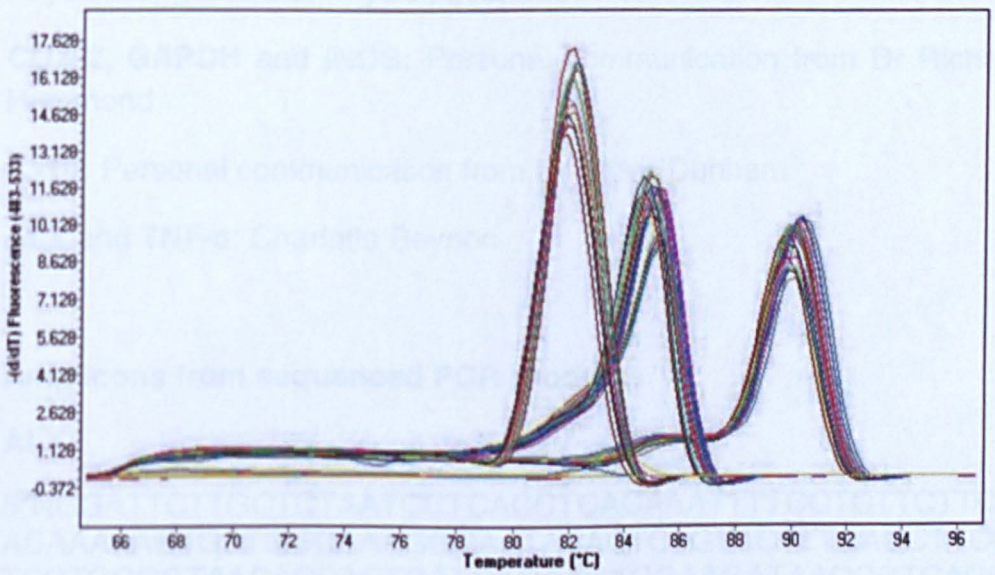
COX-2



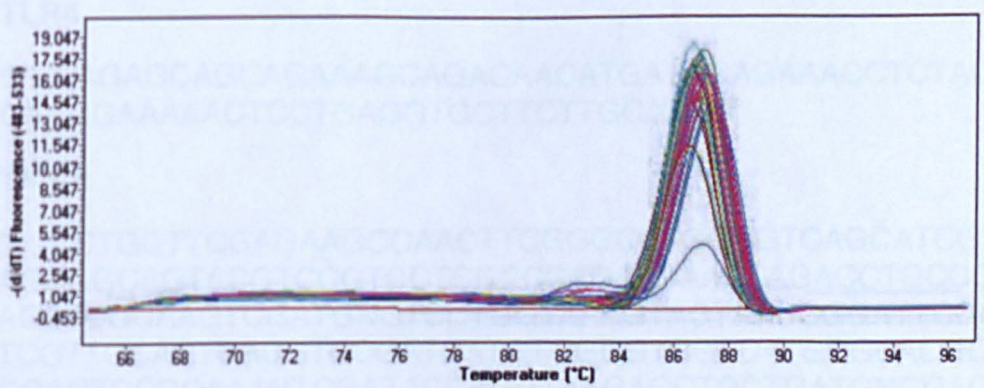
GAPDH



Beta-actin, TNF-A, IL-1B



TLR-4, INOS



Appendix 3: Primer sources and sequenced genes

Primer sources

Beta actin and 18s: ZHANG, Y. W., DAVIS, E. G. & BAI, J. (2009) Determination of internal control for gene expression studies in equine tissues and cell culture using quantitative RT-PCR. *Vet Immunol Immunopathol,* 130, 114-9. 18S, B-actin

TLR-4: BERNDT, A., DERKSEN, F. J., VENTA, P. J., EWART, S., YUZBASIYAN-GURKAN, V. & ROBINSON, N. E. (2007) Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction. *Am J Physiol Lung Cell Mol Physiol,* 292, L936-43.

COX-2, GAPDH and **iNOS:** Personal communication from Dr Richard Hammond

IL-1β: Personal communication from Dr Steve Dunham

ALX and **TNF-α**: Charlotte Beynon

Amplicons from sequenced PCR products

ALX

5"NGGATTCTTGCTCTAATCCTCACCTCACAAATTTTCCTCTTCTTCACT
ACAAAAAGTGATGGGAAAGGGAATACACTCTGTACTTTCAGCTTCGAA
TCCTGGGGTAACACCACTGATGAGAAGAGGAAGATAACCGTCACCGT
GTTGACAGTCAGAGGGATCATCCGA3"

TLR4

5"GCAGAGCAGCAGAAAGCAGACAACATGATGCAGAAACCTCTACCT
GAAGGAAAAACTCCTGAGGTGCTTCTTGCAGG3"

TNF-α

5"CCCTGGTTCGAGAAGCCAACTTCGGGCCTGGGGTCAGCATCGTGG
AGCGGCAGTACGTCCGTCCTCGCCCACACCAAGTAGACCTGCCCGG
ACTCCGCAAGTCGATGNGTCCTGGTAAAGTAGTAACCGGATTCCACT
TCGTTGCACTGACGTCCGATCATGTGGGGTCGACATGCGCACGCCC
GGACTCCGCAAAGTCGATACCAGGTAAGAGCTGCTGATGNGGAGCG
AGGACCGTGCCCCTCTACGCAGCTGAGTCGGGCTGTGCTGGACCGG
GGTCTGGGCCCCTCACGGCCGCCTGCCCGGACTCCTCAAACAT3"

INOS

5"CCAGGCTGGAAGCCGTAACAAAGGAGATAGAAACAACAGGAACCT
ACCAGCTGACGGGAGATGAGCTCATCTTTGCCACCAAGCAGGCCTG
GCGCAATGCCCCCCGCTGCATCGGGAGGATCCAGTGGTCCAACCTA
CAGGTCTTTGACGCCCGGAGCTGTTCTACTGCCCAGGAAATGTTCGA
ACACATCTGCAGACACCTGCGTTACTCCACCAACAATGGCAACATCA
GGTCGGCCATCACCGTGTTCCCCCAGCGGAGCGATGGAAAGCATGA
CTTCCGGATCTGGAATGCTCAGCTCATCCGGTATGCCGGGTACCAGA
TGCCTGATGGCAC3"

COX-2

5"ACTTACGGAGAGAAGGAAATGGCTGCGGAGTTAGAAGCACTCTAT
GGTGACATTGATGCCATGGAGCTGTATCCTGCCCTTCTGGTGGAAAA
GCCTCGCCCAGATGCCATCTTCGGGGAGACCATGGTAGAACTTGGG
GCACCATTCTCCTTGAAAGGACTTTTGGGTAATCCTATCTGTTCTCCT
GACTACTGGAAACCCAGCACTTTTGGTGGAGAAGTAGGTTTTAAAATC
ATCAACACTGCCTCAATTCAGTCTCTCANCTGCAATAAA3"

Appendix 4: List of suppliers and addresses

AbCam plc

330 Cambridge Science Park,

Cambridge

CB4 0FL

Amersham Biosciences

Amersham Pl

Amersham,

Buckinghamshire

HP7 9NA

Bio-Rad Laboratories

Bio-Rad House

Maxted Road

Hemel Hempstead

Herts

HP2 7DX

Cayman Chemicals

Cambridge Biosciences

Munro House Trafalgar Way,

Bar Hill,

Cambridge CB23 8SQ

Dako UK Ltd

Cambridge House

St Thomas Place, Ely

Cambridgeshire CB7 4EX

Fisher Scientific

Bishop Meadow Road

Loughborough

LE11 5RG

GE Healthcare

Pollards Wood

Nightingales Lane

Chalfont St Giles

Buckinghamshire

HP8 4SP

Invitrogen

Life Technologies Ltd

3 Fountain Drive

Inchinnan Business Park

Paisley PA4 9RF, UK

Lonza

Lonza Biologics plc

228 Bath Road

GB-Slough, Berkshire SL1 4DX

Polysciences,

Northampton,

Park Scientific Ltd.

24 Low Farm Place

Moulton Park

GB-NN3 6HY Northampton

Roche Diagnostics Limited

Applied Science

Charles Avenue

Burgess Hill

West Sussex

RH15 9RY

Sarstedt Ltd.

68 Boston Road

Beaumont Leys

Leicester LE4 1AW

Sigma-Aldrich

Poole

Dorset

BH12 4QH

Thermo Fisher Scientific

Stafford House

Boundary Way

Hemel Hempstead

HP2 7GEUK

Qiagen

Qiagen House,

Fleming Way,

Crawley

West Sussex

RH10 9NQ UK

Whatman plc

Springfield Mill

James Whatman Way

Maidstone

Kent

ME14 2LE